Methods and Compositions for RNA Interference

Government Support

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Related Applications

This application is a continuation-in-part of PCT application PCT/US01/08435, filed 16 March 2001, and claims the benefit of US Provisional applications USSN 60/189,739 filed 16 March 2000 and USSN 60/243,097 filed 24 October 2000. The specifications of such applications are incorporated by reference herein.

Background of the Invention

"RNA interference", "post-transcriptional gene silencing", "quelling" — these different names describe similar effects that result from the overexpression or misexpression of transgenes, or from the deliberate introduction of double-stranded RNA into cells (reviewed in Fire A (1999) Trends Genet 15: 358–363; Sharp PA (1999) Genes Dev 13: 139–141; Hunter C (1999) Curr Biol 9: R440–R442; Baulcombe DC (1999) Curr Biol 9: R599–R601; Vaucheret et al. (1998) Plant J 16: 651–659). The injection of double-stranded RNA into the nematode *Caenorhabditis elegans*, for example, acts systemically to cause the post-transcriptional depletion of the homologous endogenous RNA (Fire et al. (1998) Nature 391: 806–811; and Montgomery et al. (1998) PNAS 95: 15502–15507). RNA interference, commonly referred to as RNAi, offers a way of specifically and potently inactivating a cloned gene, and is proving a powerful tool for investigating gene function. Although the phenomenon is interesting in its own right; the mechanism has been rather mysterious, but recent research — for example that recently reported by Smardon et al. (2000) Curr Biol 10: 169–178— is beginning to shed light on the nature and evolution of the biological processes that underlie RNAi.

RNAi was discovered when researchers attempting to use the antisense RNA approach to inactivate a *C. elegans* gene found that injection of sense-strand RNA was actually as effective as the antisense RNA at inhibiting gene function (Guo et al. (1995) Cell 81: 611–620). Further investigation revealed that the active agent was modest amounts of double-stranded RNA that contaminate *in vitro* RNA preparations. Researchers quickly determined the 'rules' and effects of RNAi which have become the paradigm for thinking about the mechanism which mediates this affect. Exon sequences

are required, whereas introns and promoter sequences, while ineffective, do not appear to compromise RNAi (though there may be gene-specific exceptions to this rule). RNAi acts systemically — injection into one tissue inhibits gene function in cells throughout the animal. The results of a variety of experiments, in *C. elegans* and other organisms, indicate that RNAi acts to destabilize cellular RNA after RNA processing.

The potency of RNAi inspired Timmons and Fire (1998 Nature 395: 854) to do a simple experiment that produced an astonishing result. They fed to nematodes bacteria that had been engineered to express double-stranded RNA corresponding to the *C. elegans unc-22* gene. Amazingly, these nematodes developed a phenotype similar to that of *unc-22* mutants that was dependent on their food source. The ability to conditionally expose large numbers of nematodes to gene-specific double-stranded RNA formed the basis for a very powerful screen to select for RNAi-defective *C. elegans* mutants and then to identify the corresponding genes.

Double-stranded RNAs (dsRNAs) can provoke gene silencing in numerous in vivo contexts including *Drosophila*, *Caenorhabditis elegans*, planaria, hydra, trypanosomes, fungi and plants. However, the ability to recapitulate this phenomenon in higher eukaryotes, particularly mammalian cells, has not been accomplished in the art. Nor has the prior art demonstrated that this phenomena can be observed in cultured eukaryotic cells. Additionally, the 'rules' established by the prior art have taught that RNAi requires exon sequences, and thus constructs consisting of intronic or promoter sequences were not believed to be effective reagents in mediating RNAi. The present invention aims to address each of these deficiencies in the prior art and provides evidence both that RNAi can be observed in cultured eukaryotic cells and that RNAi constructs consisting of non-exon sequences can effectively repress gene expression.

Summary of the Invention

One aspect of the present invention provides a method for attenuating expression of a target gene in cultured cells, comprising introducing double stranded RNA (dsRNA) into the cells in an amount sufficient to attenuate expression of the target gene, wherein the dsRNA comprises a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of the target gene.

Another aspect of the present invention provides a method for attenuating expression of a target gene in a mammalian cell, comprising

(i) activating one or both of a Dicer activity or an Argonaut activity in the cell, and

(ii) introducing into the cell a double stranded RNA (dsRNA) in an amount sufficient to attenuate expression of the target gene, wherein the dsRNA comprises a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of the target gene.

In certain embodiments, the cell is suspended in culture; while in other embodiments the cell is in a whole animal, such as a non-human mammal.

In certain preferred embodiments, the cell is engineered with (i) a recombinant gene encoding a Dicer activity, (ii) a recombinant gene encoding an Argonaut activity, or (iii) both. For instance, the recombinant gene may encode, for a example, a protein which includes an amino acid sequence at least 50 percent identical to SEQ ID No. 2 or 4; or be defined by a coding sequence which hybridizes under wash conditions of 2 x SSC at 22 °C to SEQ ID No. 1 or 3. In certain embodiments, the recombinant gene may encode, for a example, a protein which includes an amino acid sequence at least 50 percent identical to the Argonaut sequence shown in Figure 24. In certain embodiments, the recombinant gene may encode a protein which includes an amino acid sequence at least 60 percent, 70 percent, 80 percent, 85 percent, 90 percent, or 95 percent identical to SEQ ID No. 2 or 4. In certain embodiments, the recombinant gene may be defined by a coding sequence which hybridizes under stringent conditions, including a wash step selected from 0.2X-2.0X SSC at from 50 °C-65 °C, to SEQ ID No. 1 or 3.

In certain embodiments, rather than use a heterologous expression construct(s), an endogenous Dicer gene or Argonaut gene can be activated, e.g, by gene activation technology, expression of activated transcription factors or other signal transduction protein(s), which induces expression of the gene, or by treatment with an endogenous factor which upregulates the level of expression of the protein or inhibits the degradation of the protein.

In certain preferred embodiments, the target gene is an endogenous gene of the cell. In other embodiments, the target gene is a heterologous gene relative to the genome of the cell, such as a pathogen gene, e.g., a viral gene.

In certain embodiments, the cell is treated with an agent that inhibits protein kinase RNA-activated (PKR) apoptosis, such as by treatment with agents which inhibit expression of PKR, cause its destruction, and/or inhibit the kinase activity of PKR.

In certain preferred embodiments, the cell is a primate cell, such as a human cell.

In certain preferred embodiments, the length of the dsRNA is at least 20, 21 or 22 nucleotides in length, e.g., corresponding in size to RNA products produced by Dicer-dependent cleavage. In certain embodiments, the dsRNA construct is at least 25, 50, 100,

200, 300 or 400 bases. In certain embodiments, the dsRNA construct is 400-800 bases in length.

In certain preferred embodiments, expression of the target gene is attenuated by at least 5 fold, and more preferably at least 10, 20 or even 50 fold, e.g., relative to the untreated cell or a cell treated with a dsRNA construct which does not correspond to the target gene.

Yet another aspect of the present invention provides a method for attenuating expression of a target gene in cultured cells, comprising introducing an expression vector having a "coding sequence" which, when transcribed, produces double stranded RNA (dsRNA) in the cell in an amount sufficient to attenuate expression of the target gene, wherein the dsRNA comprises a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of the target gene. In certain embodiments, the vector includes a single coding sequence for the dsRNA which is operably linked to (two) transcriptional regulatory sequences which cause transcription in both directions to form complementary transcripts of the coding sequence. In other embodiments, the vector includes two coding sequences which, respectively, give rise to the two complementary sequences which form the dsRNA when annealed. In still other embodiments, the vector includes a coding sequence which forms a hairpin. In certain embodiments, the vectors are episomal, e.g., and transfection is transient. In other embodiments, the vectors are chromosomally integrated, e.g., to produce a stably transfected cell line. Preferred vectors for forming such stable cell lines are described in US Patent 6,025,192 and PCT publication WO/9812339, which are incorporated by reference herein.

Another aspect of the present invention provides a method for attenuating expression of a target gene in cultured cells, comprising introducing an expression vector having a "non-coding sequence" which, when transcribed, produces double stranded RNA (dsRNA) in the cell in an amount sufficient to attenuate expression of the target gene. The non-coding sequence may include intronic or promoter sequence of the target gene of interest, and the dsRNA comprises a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of the promoter or intron of the target gene. In certain embodiments, the vector includes a single sequence for the dsRNA which is operably linked to (two) transcriptional regulatory sequences which cause transcription in both directions to form complementary transcripts of the sequence. In other embodiments, the vector includes two sequences which, respectively, give rise to the two complementary sequences which form the dsRNA when annealed. In still other embodiments, the vector includes a coding sequence which forms a hairpin. In certain embodiments, the vectors are episomal, e.g., and transfection is transient. In other embodiments, the vectors are chromosomally integrated, e.g., to produce a stably transfected cell line. Preferred vectors

for forming such stable cell lines are described in US Patent 6,025,192 and PCT publication WO/9812339, which are incorporated by reference herein.

Another aspect the present invention provides a double stranded (ds) RNA for inhibiting expression of a mammalian gene. The dsRNA comprises a first nucleotide sequence that hybridizes under stringent conditions, including a wash step of 0.2X SSC at 65 °C, to a nucleotide sequence of at least one mammalian gene and a second nucleotide sequence which is complementary to the first nucleotide sequence.

In one embodiment, the first nucleotide sequence of said double-stranded RNA is at least 20, 21, 22, 25, 50, 100, 200, 300, 400, 500, 800 nucleotides in length.

In another embodiment, the first nucleotide sequence of said double-stranded RNA is identical to at least one mammalian gene. In another embodiment, the first nucleotide sequence of said double-stranded RNA is identical to one mammalian gene. In yet another embodiment, the first nucleotide sequence of said double-stranded RNA hybridizes under stringent conditions to at least one human gene. In still another embodiment, the first nucleotide sequence of said double-stranded RNA is identical to at least one human gene. In still another embodiment, the first nucleotide sequence of said double-stranded RNA is identical to one human gene.

The double-stranded RNA may be an siRNA or a hairpin, and may be expressed transiently or stably. In one embodiment, the double-stranded RNA is a hairpin comprising a first nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence of at least one mammalian gene, and a second nucleotide sequence which is a complementary inverted repeat of said first nucleotide sequence and hybridizes to said first nucleotide sequence to form a hairpin structure.

The first nucleotide sequence of said double-stranded RNA can hybridize to either coding or non-coding sequence of at least one mammalian gene. In one embodiment, the first nucleotide sequence of said double-stranded RNA hybridizes to a coding sequence of at least one mammalian gene. In another enbodiment, the first nucleotide sequence of said double-stranded RNA hybridizes to a coding sequence of at least one human gene. In another embodiment, the first nucleotide sequence of said double-stranded RNA is identical to a coding sequence of at least one mammalian gene. In still another embodiment, the first nucleotide sequence of said double-stranded RNA is identical to a coding sequence of at least one human gene.

In another embodiment, the first nucleotide sequence of said double-stranded RNA hybridizes to a non-coding sequence of at least one mammalian gene. In another enbodiment, the first nucleotide sequence of said double-stranded RNA hybridizes to a non-coding sequence of at least one human gene. In another embodiment, the first

nucleotide sequence of said double-stranded RNA is identical to a non-coding sequence of at least one mammalian gene. In still another embodiment, the first nucleotide sequence of said double-stranded RNA is identical to a non-coding sequence of at least one human gene. In any of the foregoing embodiments, the non-coding sequence may be a non-transcribed sequence.

Still another aspect of the present invention provides an assay for identifying nucleic acid sequences, either coding or non-coding sequences, responsible for conferring a particular phenotype in a cell, comprising

(i) constructing a variegated library of nucleic acid sequences from a cell in an orientation relative to a promoter to produce double stranded DNA;

(ii) introducing the variegated dsRNA library into a culture of target cells;

(iii) identifying members of the library which confer a particular phenotype on the cell, and identifying the sequence from a cell which correspond, such as being identical or homologous, to the library member.

Yet another aspect of the present invention provides a method of conducting a drug discovery business comprising:

(i) identifying, by the subject assay, a target gene which provides a phenotypically desirable response when inhibited by RNAi;

(ii) identifying agents by their ability to inhibit expression of the target gene or the activity of an expression product of the target gene;

(iii) conducting therapeutic profiling of agents identified in step (b), or further analogs thereof, for efficacy and toxicity in animals; and

(iv) formulating a pharmaceutical preparation including one or more agents identified in step (iii) as having an acceptable therapeutic profile.

The method may include an additional step of establishing a distribution system for distributing the pharmaceutical preparation for sale, and may optionally include establishing a sales group for marketing the pharmaceutical preparation.

Another aspect of the present invention provides a method of conducting a target discovery business comprising:

(i) identifying, by the subject assay, a target gene which provides a phenotypically desirable response when inhibited by RNAi;

(ii) (optionally) conducting therapeutic profiling of the target gene for efficacy and toxicity in animals; and

(iii). licensing, to a third party, the rights for further drug development of inhibitors of the target gene.

Another aspect of the invention provides a method for inhibiting RNAi by inhibiting the expression or activity of an RNAi enzyme. Thus, the subject method may include inhibiting the activity of Dicer and/or the 22-mer RNA.

Still another aspect relates to a method for altering the specificity of an RNAi by modifying the sequence of the RNA component of the RNAi enzyme.

In another aspect, gene expression in an undifferentiated stem cell, or the differentiated progeny thereof, is altered by introducing dsRNA of the present invention. In one embodiment, the stem cells are embryonic stem cells. Preferably, the embryonic stem cells are derived from mammals, more preferably from non-human primates, and most preferably from humans.

The embryonic stem cells may be isolated by methods known to one of skill in the art from the inner cell mass (ICM) of blastocyst stage embryos. In one embodiment the embryonic stem cells are obtained from previously established cell lines. In a second embodiment, the embryonic stem cells are derived *de novo* by standard methods.

In another aspect, the embryonic stem cells are the result of nuclear transfer. The donor nuclei are obtained from any adult, fetal, or embryonic tissue by methods well known in the art. In one embodiment, the donor nuclei is transferred to a recipient oocyte which had previously been modified. In one embodiment, the oocyte is modified using one or more dsRNAs. Exemplary modifications of the recipient oocyte include any changes in gene or protein expression that prevent an embryo derived from said modified oocyte from successfully implanting in the uterine wall. Since implantation in the uterine wall is essential for fertilized mammalian embryos to progress from beyond the blastocyst stage, embryos made from such modified oocytes could not give rise to viable organisms. Non-limiting examples of such modifications include those that decrease or eliminate expression of cell surface receptors (*i.e.,* integrins) required for the recognition between the blastocyst and the uterine wall, modifications that decrease or eliminate expression of proteases (*i.e.,* collagenase, stromelysin, and plasminogen activator) required to digest matrix in the uterine lining and thus allow proper implantation, and modifications that decrease or eliminate expression of proteases (i.e., strypsin) necessary for the blastocyst to hatch from the zona pellucida. Such hatching is required for implantation.

In another embodiment, embryonic stem cells, embryonic stem cells obtained from fertilization of modified oocytes, or the differentiated progeny thereof, can be modified or further modified with one or more dsRNAs. In a preferred embodiment, the modification decreases or eliminates MHC expression. Cells modified in this way will be tolerated by the recipient, thus avoiding complications arising from graft rejection. Such modified cells are suitable for transplantation into a related or unrelated patient to treat a condition characterized by cell damage or cell loss.

In another aspect of the invention, the undifferentiated stem cell is an adult stem cell. Exemplary adult stem cells include, but are not limited to, hematopoietic stem cells, mesenchymal stem cells, cardiac stem cells, pancreatic stem cells, and neural stem cells. Exemplary adult stem cells include any stem cell capable of forming differentiated ectodermal, mesodermal, or endodermal derivatives. Non-limiting examples of differentiated cell types which arise from adult stem cells include: blood, skeletal muscle, myocardium, endocardium, pericardium, bone, cartilage, tendon, ligament, connective tissue, adipose tissue, liver, pancreas, skin, neural tissue, lung, small intestine, large intestine, gall bladder, rectum, anus, bladder, female or male reproductive tract, genitals, and the linings of the body cavity.

In one embodiment, an undifferentiated adult stem cell, or the differentiated progeny thereof, is altered with one or more dsRNAs to decrease or eliminate MHC expression. Cells modified in this way will be tolerated by the recipient, thus avoiding complications arising from graft rejection. Such modified cells are suitable for transplantation into a related or unrelated patient to treat a condition characterized by cell damage or cell loss.

In another aspect of the invention, an embryonic stem cell, an undifferentiated adult stem cell, or the differentiated progeny of either an embryonic or adult stem cell is altered with one or more dsRNA to decrease or eliminate expression of genes required for HIV infection. In a preferred embodiment, the stem cell is one capable of giving rise to hematopoietic cells. Modified cells with hematopoietic potential can be transplanted into a patient as a preventative therapy or treatment for HIV or AIDS.

Another aspect of the invention relates to purified or semi-purified preparations of the RNAi enzyme or components thereof. In certain embodiments, the preparations are used for identifying compounds, especially small organic molecules, which inhibit or potentiate the RNAi activity. Small molecule inhibitors, for example, can be used to

inhibit dsRNA responses in cells which are purposefully being transfected with a virus which produces double stranded RNA.

The dsRNA construct may comprise one or more strands of polymerized ribonucleotide. It may include modifications to either the phosphate-sugar backbone or the nucleoside. The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The dsRNA construct may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses of double-stranded material may yield more effective inhibition. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition. In certain embodiments, dsRNA constructs containing a nucleotide sequences identical to a portion of the target gene are preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence (i.e., RNA sequences similar to the target sequence) have also been found to be effective for inhibition. Thus, sequence identity may be optimized by alignment algorithms known in the art and calculating the percent difference between the nucleotide sequences. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript. In another embodiment, dsRNA constructs containing nucleotide sequences identical to a non-coding portion of the target gene are preferred for inhibition. Exemplary non-coding regions include introns and the promoter region. Sequences with insertions, deletions, and single point mutations relative to the target non-coding sequence may also be used.

Yet another aspect of the invention pertains to transgenic non-human mammals which include a transgene encoding a dsRNA construct, wherein the dsRNA is identical or similar to either the coding or non-coding sequence of the target gene, preferably which is stably integrated into the genome of cells in which it occurs. The animals can be derived by oocyte microinjection, for example, in which case all of the nucleated cells of the animal will include the transgene, or can be derived using embryonic stem (ES) cells which have been transfected with the transgene, in which case the animal is a chimera and only a portion of its nucleated cells will include the transgene. In certain instances, the sequence-independent dsRNA response, e.g., the PKR response, is also inhibited in those cells including the transgene.

In still other embodiments, dsRNA itself can be introduced into an ES cell in order to effect gene silencing, and that phenotype will be carried for at least several rounds of division, e.g., into the progeny of that cell.

Brief Description of the Drawings

Figure 1: RNAi in S2 cells. (**a**) *Drosophila* S2 cells were transfected with a plasmid that directs *lacZ* expression from the copia promoter in combination with dsRNAs corresponding to either human CD8 or lacZ, or with no dsRNA, as indicated. (**b**) S2 cells were co-transfected with a plasmid that directs expression of a GFP–US9 fusion protein and dsRNAs of either *lacZ* or *cyclin E*, as indicated. Upper panels show FACS profiles of the bulk population. Lower panels show FACS profiles from GFP-positive cells. (**c**) Total RNA was extracted from cells transfected with *lacZ*, *cyclin E*, *fizzy* or *cyclin A* dsRNAs, as indicated. Northern blots were hybridized with sequences not present in the transfected dsRNAs.

Figure 2: RNAi *in vitro*. (**a**) Transcripts corresponding to either the first 600 nucleotides of *Drosophila cyclin E* (E600) or the first 800 nucleotides of *lacZ* (Z800) were incubated in lysates derived from cells that had been transfected with either *lacZ* or *cyclin E* (cycE) dsRNAs, as indicated. Time points were 0, 10, 20, 30, 40 and 60 min for *cyclin E* and 0, 10, 20, 30 and 60 min for *lacZ*. (**b**) Transcripts were incubated in an extract of S2 cells that had been transfected with *cyclin E* dsRNA (cross-hatched box, below). Transcripts corresponded to the first 800 nucleotides of *lacZ* or the first 600, 300, 220 or 100 nucleotides of *cyclin E*, as indicated. Eout is a transcript derived from the portion of the *cyclin E* cDNA not contained within the transfected dsRNA. E-ds is identical to the dsRNA that had been transfected into S2 cells. Time points were 0 and 30 min. (**c**) Synthetic transcripts complementary to the complete *cyclin E* cDNA (Eas) or the final 600 nucleotides (Eas600) or 300 nucleotides (Eas300) were incubated in extract for 0 or 30 min.

Figure 3: Substrate requirements of the RISC. Extracts were prepared from cells transfected with *cyclin E* dsRNA. Aliquots were incubated for 30 min at 30 °C before the addition of either the *cyclin E* (E600) or *lacZ* (Z800) substrate. Individual 20 µl aliquots, as indicated, were pre-incubated with 1 mM $CaCl_2$ and 5 mM EGTA, 1 mM $CaCl_2$, 5 mM EGTA and 60 U of micrococcal nuclease, 1 mM $CaCl_2$ and 60 U of micrococcal nuclease or 10 U of DNase I (Promega) and 5 mM EGTA. After the 30 min pre-incubation, EGTA was added to those samples that lacked it. Yeast tRNA (1 µg) was added to all samples. Time points were at 0 and 30 min.

Figure 4: The RISC contains a potential guide RNA. (**a**) Northern blots of RNA from either a crude lysate or the S100 fraction (containing the soluble nuclease activity, see Methods) were hybridized to a riboprobe derived from the sense strand of the *cyclin E* mRNA. (**b**) Soluble *cyclin-E*-specific nuclease activity was fractionated as described in Methods. Fractions from the anion-exchange resin were incubated with the lacZ, control

substrate (upper panel) or the cyclin E substrate (centre panel). Lower panel, RNA from each fraction was analysed by northern blotting with a uniformly labelled transcript derived from sense strand of the *cyclin E* cDNA. DNA oligonucleotides were used as size markers.

Figure 5: Generation of 22mers and degradation of mRNA are carried out by distinct enzymatic complexes. (**a**) Extracts prepared either from 0-12 hour *Drosophila* embryos or *Drosophila* S2 cells (see Methods) were incubated for 0, 15, 30, or 60 minutes (left to right) with a uniformly-labeled double-stranded RNA corresponding to the first 500 nucleotides of the *Drosophila cyclin E* coding region. M indicates a marker prepared by *in vitro* transcription of a synthetic template. The template was designed to yield a 22 nucleotide transcript. The doublet most probably results from improper initiation at the +1 position. (**b**) Whole-cell extracts were prepared from S2 cells that had been transfected with a dsRNA corresponding to the first 500 nt. of the luciferase coding region. S10 extracts were spun at 30,000xg for 20 minutes which represents our standard RISC extract. S100 extracts were prepared by further centrifugation of S10 extracts for 60 minutes at 100,000xg. Assays for mRNA degradation were carried out as described previously for 0, 30 or 60 minutes (left to right in each set) with either a single-stranded luciferase mRNA or a single-stranded cyclin E mRNA, as indicated. (**c**) S10 or S100 extracts were incubated with cyclin E dsRNAs for 0, 60 or 120 minutes (L to R).

Figure 6: Production of 22mers by recombinant CG4792/Dicer. (**a**) *Drosophila* S2 cells were transfected with plasmids that direct the expression of T7-epitope tagged versions of Drosha, CG4792/Dicer-1 and Homeless. Tagged proteins were purified from cell lysates by immunoprecipitation and were incubated with *cyclin E* dsRNA. For comparison, reactions were also performed in *Drosophila* embryo and S2 cell extracts. As a negative control, immunoprecipitates were prepared from cells transfected with a β-galactosidase expression vector. Pairs of lanes show reactions performed for 0 or 60 minutes. The synthetic marker (M) is as described in the legend to Figure 1. (**b**) Diagrammatic representations of the domain structures of CG4792/Dicer-1, Drosha and Homeless are shown. (**c**) Immunoprecipitates were prepared from detergent lysates of S2 cells using an antiserum raised against the C-terminal 8 amino acids of Drosophila Dicer-1 (CG4792). As controls, similar preparations were made with a pre-immune serum and with an immune serum that had been pre-incubated with an excess of antigenic peptide. Cleavage reactions in which each of these precipitates was incubated with an ~500 nt. fragment of Drosophila cyclin E are shown. For comparsion, an incubation of the substrate in Drosophila embryo extract was electrophoresed in parallel. (**d**) Dicer immunoprecipitates were incubated with dsRNA substrates in the presence or absence of ATP. For comparison, the same substrate was incubated with S2 extracts that either contained added ATP or that were depleted of ATP using glucose and hexokinase (see

methods). (e) Drosophila S2 cells were transfected with uniformly, ^{32}P-labelled dsRNA corresponding to the first 500 nt. of GFP. RISC complex was affinity purified using a histidine-tagged version of *Drosophila* Ago-2, a recently identified component of the RISC complex (Hammond et al., in prep). RISC was isolated either under conditions in which it remains ribosome associated (ls, low salt) or under conditions that extract it from the ribosome in a soluble form (hs, high salt). For comparison, the spectrum of labelled RNAs in the total lysate is shown. (f) Guide RNAs produced by incubation of dsRNA with a Dicer immunoprecipitate are compared to guide RNAs present in an affinity-purified RISC complex. These precisely comigrate on a gel that has single-nucleotide resolution. The lane labelled control is an affinity selection for RISC from a cell that had been transfected with labeled dsRNA but not with the epitope-tagged *Drosophila* Ago-2.

Figure 7: Dicer participates in RNAi. (a) Drosophila S2 cells were transfected with dsRNAs corresponding to the two Drosophila Dicers (CG4792 and CG6493) or with a control dsRNA corresponding to murine caspase 9. Cytoplasmic extracts of these cells were tested for Dicer activity. Transfection with Dicer dsRNA reduced activity in lysates by 7.4-fold. (b) The Dicer-1 antiserum (CG4792) was used to prepare immunoprecipitates from S2 cells that had been treated as described above. Dicer dsRNA reduced the activity of Dicer-1 in this assay by 6.2-fold. (c) Cells that had been transfected two days previously with either mouse caspase 9 dsRNA or with Dicer dsRNA were cotransfected with a GFP expression plasmid and either control, luciferase dsRNA or GFP dsRNA. Three independent experiments were quantified by FACS. A comparison of the relative percentage of GFP-positive cells is shown for control (GFP plasmid plus luciferase dsRNA) or silenced (GFP plamsid plus GFP dsRNA) populations in cells that had previously been transfected with either control (caspase 9) or Dicer dsRNAs.

Figure 8: Dicer is an evolutionarily conserved ribonuclease. (a) A model for production of 22mers by Dicer. Based upon the proposed mechanism of action of Ribonuclease III, we propose that Dicer acts on its substrate as a dimer. The positioning of the two ribonuclease domains (RIIIa and RIIIb) within the enzyme would thus determine the size of the cleavage product. An equally plausible alternative model could be derived in which the RIIIa and RIIIb domains of each Dicer enzyme would cleave in concert at a single position. In this model, the size of the cleavage product would be determined by interaction between two neighboring Dicer enzymes. (b) Comparison of the domain structures of potential Dicer homologs in various organisms (*Drosophila* - CG4792, CG6493, *C. elegans* - K12H4.8, *Arabidopsis* – CARPEL FACTORY, T25K16.4, AC012328_1, human Helicase-MOI and *S. pombe* - YC9A_SCHPO). The ZAP domains were identified both by analysis of individual sequences with Pfam and by Psi-blast searches. The ZAP domain in the putative *S. pombe* Dicer is not detected by PFAM but is identified by Psi-Blast and is thus shown in a different color. For

comparison, a domain structure of the RDE1/QDE2/ARGONAUTE family is shown. It should be noted that the ZAP domains are more similar within each of the Dicer and ARGONAUTE families than they are between the two groups. (c) An alignment of the ZAP domains in selected Dicer and Argonaute family members is shown. The alignment was produced using ClustalW.

Figure 9: Purification strategy for RISC. (second step in RNAi model).

Figure 10: Fractionation of RISC activity over sizing column. Activity fractionates as 500 KDa complex. Also, antibody to *Drosophila* argonaute 2 cofractionates with activity.

Figure 11-13: Fractionation of RISC over monoS, monoQ, Hydroxyapatite columns. *Drosophila* argonaute 2 protein also cofactionates.

Figure 14: Alignment of *Drosophila* argonaute 2 with other family members.

Figure 15: Confirmation of *Drosophila* argonaute 2. S2 cells were transfected with labeled dsRNA and His tagged argonaute. Argonaute was isolated on nickel agarose and RNA component was identified on 15% acrylamide gel.

Figure 16: S2 cell and embryo extracts were assayed for 22mer generating activity.

Figure 17: RISC can be separated from 22mer generating activity (dicer). Spinning extracts (S100) can clear RISC activity from supernatant (left panel) however, S100 spins still contain dicer activity (right panel).

Figure 18: Dicer is specific for dsRNA and prefers longer substrates.

Figure 19: Dicer was fractionated over several columns.

Figure 20: Identification of dicer as enzyme which can process dsRNA into 22mers. Various RNaseIII family members were expressed with n terminal tags, immunoprecipitated, and assayed for 22mer generating activity (left panel). In right panel, antibodies to dicer could also precipitate 22mer generating activity.

Figure 21: Dicer requires ATP.

Figure 22: Dicer produces RNAs that are the same size as RNAs present in RISC.

Figure 23: Human dicer homolog when expressed and immunoprecipitated has 22mer generating activity.

Figure 24: Sequence of *Drosophila* argonaute 2. Peptides identified by microsequencing are shown in underline.

Figure 25: Molecular charaterization of *Drosophila* argonaute 2. The presence of an intron in coding sequence was determined by northern blotting using intron probe. This results in a different 5' reading frame then the published genome seqeunce. Number of polyglutamine repeats was determined by genomic PCR.

Figure 26: Dicer activity can be created in human cells by expression of human dicer gene. Host cell was 293. Crude extracts had dicer activity, while activity was absent from untransfected cells. Activity is not dissimilar to that seen in drosophila embryo extracts.

Figure 27: A ~500 nt. fragment of the gene that is to be silenced (X) is inserted into the modified vector as a stable direct repeat using standard cloning procedures. Treatment with commercially available cre recombinase reverses sequences within the loxP sites (L) to create an inverted repeat. This can be stably maintained and amplified in an sbc mutant bacterial strain (DL759). Transcription in vivo from the promoter of choice (P) yields a hairpin RNA that causes silencing. A zeocin resistance marker is included to insure maintenance of the direct and inverted repeat structures; however this is non-essential in vivo and could be removed by pre-mRNA splicing if desired. (Smith et al. (2000) Nature 407: 319-20).

Figure 28: The panels at the right show expression of either RFP or GFP following transient transfection into wild type P19 cells. The panels at the left demonstrate the specific suppression of GFP expression in P19 clones which stably express a 500 nt double stranded GFP hairpin. P19 clones which stably express the double stranded GFP hairpin were transiently transfected with RFP or GFP, and expression of RFP or GFP was assessed by visual inspection.

Figure 29: Hela, Chinese hamster ovary, and P19 (pluripotent, mouse embryonic carcinoma) cell lines transfected with plasmids expressing Photinus pyralis (firefly) and Renilla reniformis (sea pansy) luciferases and with dsRNA 500mers (400ng), homologous to either firefly luciferase mRNA (dsLUC) or non-homologous (dsGFP). Dual luciferase assays were carried out using an Analytical Scientific Instruments model 3010 Luminometer. In this assay Renilla luciferase serves as an internal control for dsRNA-specific suppression of firefly luciferase activity. These data demonstrate that 500mer dsRNA can specifically suppress cognate gene expression in vivo.

Figure 30: Mouse embryonic stem cells (ES cells) were transfected with plasmids expressing Photinus pyralis (firefly) and Renilla reniformis (sea pansy) luciferases and with dsRNA 500mers (400ng), homologous to either firefly luciferase mRNA (dsLUC) or non-homologous (dsGFP). Dual luciferase assays were carried out using an Analytical Scientific Instruments model 3010 Luminometer. In this assay firefly luciferase serves as an internal control for dsRNA-specific suppression of Renilla luciferase activity. These

data demonstrate that 500mer dsRNA can specifically suppress cognate gene expression in vivo.

Figure 31: P19 (a pluripontent, mouse embryonic cell line) cells transfected with plasmids expressing Photinus pyralis (firefly) and Renilla reniformis (sea pansy) luciferases and with dsRNA 500mers (500ng), homologous to either firefly luciferase mRNA (dsLUC) or non-homologous (dsGFP). Dual luciferase assays were carried out using an Analytical Scientific Instruments model 3010 Luminometer. In this assay Renilla luciferase serves as an internal control for dsRNA-specific suppression of firefly luciferase activity. These data further demonstrate that 500mer dsRNA can specifically suppress cognate gene expression in vivo and that the effect is stable over time.

Figure 32: S10 fractions from P19 cell lysates were used for in vitro translations of mRNA coding for Photinus pyralis (firefly) and Renilla reniformis (sea pansy) luciferases. Translation reactions were programmed with various amounts of dsRNA 500mers, either homologous to firefly luciferase mRNA (dsLUC) or non-homologous (dsGFP). Reactions were carried out at 30 °C for 1 hour, after which dual luciferase assays were carried out using an Analytical Scientific Instruments model 3010 Luminometer. In this assay Renilla luciferase serves as an internal control for dsRNA-specific suppression of firefly luciferase activity. These data demonstrate that 500mer dsRNA can specifically suppress cognate gene expression in vitro in a manner consistent with post-transcriptional gene silencing. Anti-sense firefly RNA did not differ significantly from dsGFP control (approximately 10%) (data not shown).

Figure 33: Provides additional evidence that stable dsRNA suppresses gene expression in vivo in a manner consistant with post-transcriptional gene silencing. P19 cells were stably transfected with a construct expressing a long dsRNA specific for GFP. Cells were then transiently transfected with a plasmind expressing GFP or with both a plasmid expressing GFP and a plasmind expressing dsRNA specific for Dicer.

Figure 34: S10 fractions from P19 cell lysates were used for in vitro translations of mRNA coding for Photinus pyralis (firefly) and Renilla reniformis (sea pansy) luciferases. Translation reactions were programmed with dsRNA, ssRNA, or asRNA 500mers, either complementary to firefly luciferase mRNA (dsFF, ssFF, or asFF), complementary to Renilla luciferase (dsREN, ssREN, or asREN) or non-complementary (dsGFP). Reactions were carried out at 30 °C for 1 hour, after a 30 min preincubation with dsRNA, ssRNA,or asRNA. Dual luciferase assays were carried out using an Analytical Scientific Instruments model 3010 Luminometer. On the left, Renilla luciferase serves as an internal control for dsRNA-specific suppression of firefly luciferase activity. On the right, firefly luciferase serves as an internal control for dsRNA-specific suppression of Renilla luciferase activity. These data demonstrate that 500mer double-stranded RNA (dsRNA) but not single-

stranded (ssRNA) or anti-sense RNA (asRNA) suppresses cognate gene expression in vitro in a manner consistent with post-transcriptional gene silencing.

Figure 35: P19 cells were grown in 6-well tissue culture plates to approximately 60% confluence. Various amounts of dsRNA, either homologous to firefly luciferase mRNA (dsLUC) or non-homologous (dsGFP), were added to each well and incubated for 12hrs under normal tissue culture conditions. Cells were then transfected with plasmids expressing Photinus pyralis (firefly) and Renilla reniformis (sea pansy) luciferases and with dsRNA 500mers (500ng). Dual luciferase assays were carried out 12 hrs post-transfection using an Analytical Scientific Instruments model 3010 Luminometer. In this assay Renilla luciferase serves as an internal control for dsRNA-specific suppression of firefly luciferase activity. These data show that 500mer dsRNA can specifically suppress cognate gene expression in vivo without transfection under normal tissue culture conditions.

Figure 36: Previous methods for generating siRNAs required costly chemical synthesis. The invention provides an in vitro method for synthesizing siRNAs using standard RNA transcription reactions.

Figure 37: Depicts three types of short RNAs corresponding to the coding region of firefly luciferase. The three types of RNAs are siRNAs, let-7 like hairpins, and simple hairpins.

Figure 38: The three types of short RNAs depicted in Figure 37 were analyzed in Drosophila S2 cells for their ability to specifically suppress firefly luciferase gene expression. All three short RNAs (siRNA, let-7 like hairpin, and simple hairpin) specifically suppress firefly luciferase gene expression.

Figure 39: The three types of short RNAs depicted in Figure 37 were analyzed in human 293T cells for their ability to specifically suppress firefly luciferase gene expression. All three short RNAs (siRNA, let-7 like hairpin, and simple hairpin) specifically suppress firefly luciferase gene expression.

Figure 40: The three types of short RNAs depicted in Figure 37 were analyzed in human HeLa cells for their ability to specifically suppress firefly luciferase gene expression. All three short RNAs (siRNA, let-7 like hairpin, and simple hairpin) specifically suppress firefly luciferase gene expression.

Figure 41: A mixture of two short hairpins, both corresponding to firefly luciferase, does not result in a synergistic suppression of gene expression. Suppression of firefly luciferase gene expression resulting from transfection of a mixture of two different short hairpins (HP #1 and HP #2) was examined. The mixture of HP #1 and HP #2 did not

have a more robust effect on the suppression of firefly luciferase gene expression than expression of HP #1 alone.

Figure 42: Encoded short hairpins specifically suppress gene expression in vivo. DNA oligonucleotides encoding 29 nucleotide hairpins corresponding to firefly luciferase were inserted into a vector containing the U6 promoter. Three independent constructs were examined for their ability to specifically suppress firefly luciferase gene expression in 293T cells. siOligo1-2, siOligo1-6, and siOligo1-19 (construct in the correct orientation) each suppressed gene expression as effectively as siRNA. In contrast, siOligo1-10 (construct in the incorrect orientation) did not suppress gene expression. An independent construct targeted to a different portion of the firefly luciferase gene did not effectively suppress gene expression in either orientation (siOligo2-23, siOligo2-36).

Figures 43-45: Strategies for stable expression of short dsRNAs.

Figure 46: Dual luciferase assays were performed as described in detail in figures 28-35, however the cells used in these experiments were PKR-/- murine embryonic fibroblasts (MEFs). Briefly, RNAi using long dsRNAs typically envokes a non-specific response in MEFs (due to PKR activity). To evaluate the effect of long dsRNA constructs to specifically inhibit gene expression in MEFs, RNAi was examined in PKR -/- MEFs. Such cells do not respond to dsRNA with a non-specific response. The data summarized in this figure demonstrates that in the absence of the non-specific PKR response, long dsRNA constructs specifically suppress gene expression in MEFs.

Figure 47: Is a schematic representation of the mouse tyrosinase promoter. Primers were used to amplify three separate regions in the proximal promoter, or to amplify sequence corresponding to an enhancer located approximately 12 kb upstream.

Detailed Description of Certain Preferred Embodiments

I. Overview

The present invention provides methods for attenuating gene expression in a cell using gene-targeted double stranded RNA (dsRNA). The dsRNA contains a nucleotide sequence that hybridizes under physiologic conditions of the cell to the nucleotide sequence of at least a portion of the gene to be inhibited (the "target" gene). The nucleotide sequence can hybridize to either coding or non-coding sequence of the target gene.

A significant aspect to certain embodiments of the present invention relates to the demonstration in the present application that RNAi can in fact be accomplished both in

cultured mammalian cells and in whole organisms. This had not been previously described in the art.

Another salient feature of the present invention concerns the ability to carry out RNAi in higher eukaryotes, particularly in non-oocytic cells of mammals, e.g., cells from adult mammals as an example.

Furthermore, in contrast to the teachings of the prior art, we demonstrate that RNAi in mammalian systems can be mediated with dsRNA identical or similar to non-coding sequence of a target gene. It was previously believed that although dsRNA identical or similar to non-coding sequences (i.e., promoter, enhancer, or intronic sequences) did not inhibit RNAi, such dsRNAs were not thought to mediate RNAi.

As described in further detail below, the present invention(s) are based on the discovery that the RNAi phenomenum is mediated by a set of enzyme activities, including an essential RNA component, that are evolutionarily conserved in eukaryotes ranging from plants to mammals.

One enzyme contains an essential RNA component. After partial purification, a multi-component nuclease (herein "RISC nuclease") co-fractionates with a discrete, 22-nucleotide RNA species which may confer specificity to the nuclease through homology to the substrate mRNAs. The short RNA molecules are generated by a processing reaction from the longer input dsRNA. Without wishing to be bound by any particular theory, these 22mer guide RNAs may serve as guide sequences that instruct the RISC nuclease to destroy specific mRNAs corresponding to the dsRNA sequences.

As illustrated, double stranded forms of the 22-mer guide RNA can be sufficient in length to induce sequence-dependent dsRNA inhibition of gene expression. In the illustrated example, dsRNA contructs are administered to cells having a recombinant luciferase reporter gene. In the control cell, e.g., no exogeneously added RNA, the level of expression of the luciferase reporter is normalized to be the value of "1". As illustrated, both long (500-mer) and short (22-mer) dsRNA constructs complementary to the luciferase gene could inhibit expression of that gene product relative to the control cell. On the other hand, similarly sized dsRNA complementary to the coding sequence for another protein, green fluorescence protein (GFP), did not significantly effect the expression of luciferase –indicating that the inhibitory phenomena was in each case sequence-dependent. Likewise, single stranded 22-mers of luciferase did not inhibit expression of that gene – indicating that the inhibitory phenomena is double stranded-dependent.

The appended examples also identify an enzyme, Dicer, that can produce the putative guide RNAs. Dicer is a member of the RNAse III family of nucleases that

specifically cleave dsRNA and is evolutionarily conserved in worms, flies, plants, fungi and, as described herein, mammals. The enzyme has a distinctive structure which includes a helicase domain and dual RNAse III motifs. Dicer also contains a region of homology to the RDE1/QDE2/ARGONAUTE family, which have been genetically linked to RNAi in lower eukaryotes. Indeed, activation of, or overexpression of Dicer may be sufficient in many cases to permit RNA interference in otherwise non-receptive cells, such as cultured eukaryotic cells, or mammalian (non-oocytic) cells in culture or in whole organisms.

In certain embodiments, the cells can be treated with an agent(s) that inhibits the general double-stranded RNA response(s) by the host cells, such as may give rise to sequence-independent apoptosis. For instance, the cells can be treated with agents that inhibit the dsRNA-dependent protein kinase known as PKR (protein kinase RNA-activated). Double stranded RNAs in mammalian cells typically activate protein kinase PKR and lead to apoptosis. The mechanism of action of PKR includes phosphorylation and inactivation of eIF2α (Fire (1999) Trends Genet 15: 358). It has also been reported that induction of NF-κB by PKR is involved in apoptosis commitment and this process is mediated through activation of the IKK complex. This sequence-independent response may reflect a form of primitive immune response, since the presence of dsRNA is a common feature of many viral lifecycles.

As described herein, Applicants have demonstrated that the PKR response can be overcome in favor of the sequence-specific RNAi response. However, in certain instances, it may be desirable to treat the cells with agents which inhibit expression of PKR, cause its destruction, and/or inhibit the kinase activity of PKR, and such methods are specifically contemplated for use in the present invention. Likewise, overexpression of agents which ectopically activate eIF2α can be used. Other agents which can be used to suppress the PKR response include inhibitors of IKK phosphorylation of IκB, inhibitors of IκB ubiquitination, inhibitors of IκB degradation, inhibitors of NF-κB nuclear translocation, and inhibitors of NF-κB interaction with κB response elements.

Other inhibitors of sequence-independent dsRNA response in cells include the gene product of the vaccinia virus E3L. The E3L gene product contains two distinct domains. A conserved carboxy-terminal domain has been shown to bind double-stranded RNA (dsRNA) and inhibit the antiviral dsRNA response by cells. Expression of at least that portion of the E3L gene in the host cell, or the use of polypeptide or peptidomimetics thereof, can be used to suppress the general dsRNA response. Caspase inhibitors sensitize cells to killing by double-stranded RNA. Accordingly, ectopic expression or activation of caspases in the host cell can be used to suppress the general dsRNA response.

In other embodiments, the subject method is carried out in cells which have little or no general response to double stranded RNA, e.g., have no PKR-dependent dsRNA

response, at least under the culture conditions. As illustrated in Figures 28-32, CHO and P19 cells can be used without having to inhibit PKR or other general dsRNA responses.

Thus, the present invention provides a process and compositions for inhibiting expression of a target gene in a cell, especially a mammalian cell. In certain embodiments, the process comprises introduction of RNA (the "dsRNA construct") with partial or fully double-stranded character into the cell or into the extracellular environment. Inhibition is specific in that a nucleotide sequence from a portion of the target gene is chosen to produce the dsRNA construct. The dsRNA may be identical or similar to coding or non-coding sequence of the target gene. In preferred embodiments, the method utilizes a cell in which Dicer and/or Argonaute activities are recombinantly expressed or otherwise ectopically activated. This process can be (1) effective in attenuating gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

II. Definitions

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a genomic integrated vector, or "integrated vector", which can become integrated into the chromsomal DNA of the host cell. Another type of vector is an episomal vector, i.e., a nucleic acid capable of extra-chromosomal replication. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". In the present specification, "plasmid" and "vector" are used interchangeably unless otherwise clear from the context.

As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as applicable to the embodiment being described, single-stranded (such as sense or antisense) and double-stranded polynucleotides.

As used herein, the term "gene" or "recombinant gene" refers to a nucleic acid comprising an open reading frame encoding a polypeptide of the present invention, including both exon and (optionally) intron sequences. The nucleic acid may also optionally include non-coding sequences such as promoter or enhancer sequences. A "recombinant gene" refers to nucleic acid encoding such regulatory polypeptides, that may optionally include intron sequences that are derived from chromosomal DNA. The term

"intron" refers to a DNA sequence present in a given gene that is not translated into protein and is generally found between exons.

A "protein coding sequence" or a sequence that "encodes" a particular polypeptide or peptide, is a nucleic acid sequence that is transcribed (in the case of DNA) and is translated (in the case of mRNA) into a polypeptide in vitro or in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA from procaryotic or eukaryotic mRNA, genomic DNA sequences from procaryotic or eukaryotic DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the coding sequence.

Likewise, "encodes", unless evident from its context, will be meant to include DNA sequences that encode a polypeptide, as the term is typically used, as well as DNA sequences that are transcribed into inhibitory antisense molecules.

The term "loss-of-function", as it refers to genes inhibited by the subject RNAi method, refers to a diminishment in the level of expression of a gene(s) in the presence of one or more dsRNA construct(s) when compared to the level in the absense of such dsRNA construct(s).

The term "expression" with respect to a gene sequence refers to transcription of the gene and, as appropriate, translation of the resulting mRNA transcript to a protein. Thus, as will be clear from the context, expression of a protein coding sequence results from transcription and translation of the coding sequence.

"Cells," "host cells" or "recombinant host cells" are terms used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

The term "cultured cells" refers to cells suspended in culture, e.g., dispersed in culture or in the form tissue. It does not, however, include oocytes or whole embryos (including blastocysts and the like) which may be provided in culture. In certain embodiments, the cultured cells are adults cells, e.g., non-embryonic.

By "recombinant virus" is meant a virus that has been genetically altered, e.g., by the addition or insertion of a heterologous nucleic acid construct into the particle.

As used herein, the terms "transduction" and "transfection" are art recognized and mean the introduction of a nucleic acid, e.g., an expression vector, into a recipient cell by

nucleic acid-mediated gene transfer. "Transformation", as used herein, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA, and, for example, the transformed cell expresses a dsRNA contruct.

"Transient transfection" refers to cases where exogenous DNA does not integrate into the genome of a transfected cell, e.g., where episomal DNA is transcribed into mRNA and translated into protein.

A cell has been "stably transfected" with a nucleic acid construct when the nucleic acid construct is capable of being inherited by daughter cells.

As used herein, a "reporter gene construct" is a nucleic acid that includes a "reporter gene" operatively linked to at least one transcriptional regulatory sequence. Transcription of the reporter gene is controlled by these sequences to which they are linked. The activity of at least one or more of these control sequences can be directly or indirectly regulated by the target receptor protein. Exemplary transcriptional control sequences are promoter sequences. A reporter gene is meant to include a promoter-reporter gene construct that is heterologously expressed in a cell.

As used herein, "transformed cells" refers to cells that have spontaneously converted to a state of unrestrained growth, i.e., they have acquired the ability to grow through an indefinite number of divisions in culture. Transformed cells may be characterized by such terms as neoplastic, anaplastic and/or hyperplastic, with respect to their loss of growth control. For purposes of this invention, the terms "transformed phenotype of malignant mammalian cells" and "transformed phenotype" are intended to encompass, but not be limited to, any of the following phenotypic traits associated with cellular transformation of mammalian cells: immortalization, morphological or growth transformation, and tumorigenicity, as detected by prolonged growth in cell culture, growth in semi-solid media, or tumorigenic growth in immuno-incompetent or syngeneic animals.

As used herein, "proliferating" and "proliferation" refer to cells undergoing mitosis.

As used herein, "immortalized cells" refers to cells that have been altered via chemical, genetic, and/or recombinant means such that the cells have the ability to grow through an indefinite number of divisions in culture.

The "growth state" of a cell refers to the rate of proliferation of the cell and the state of differentiation of the cell.

III. Exemplary Embodiments of Isolation Method

One aspect of the invention provides a method for potentiating RNAi by induction or ectopic activation of an RNAi enzyme in a cell (in vivo or in vitro) or cell-free mixtures. In preferred embodiments, the RNAi activity is activated or added to a mammalian cell, e.g., a human cell, which cell may be provided in vitro or as part of a whole organism. In other embodiments, the subject method is carried out using eukaryotic cells generally (except for oocytes) in culture. For instance, the Dicer enzyme may be activated by virtue of being recombinantly expressed or it may be activated by use of an agent which (i) induces expression of the endogenous gene, (ii) stabilizes the protein from degradation, and/or (iii) allosterically modifies the enzyme to increase its activity (by altering its Kcat, Km or both).

A. Dicer and Argonaut Activities

In certain embodiments, at least one of the activated RNAi enzymes is Dicer, or a homolog thereof. In certain preferred embodiments, the present method provides for ectopic activation of Dicer. As used herein, the term "Dicer" refers to a protein which (a) mediates an RNAi response and (b) has an amino acid sequence at least 50 percent identical, and more preferably at least 75, 85, 90 or 95 percent identical to SEQ ID No. 2 or 4, and/or which can be encoded by a nucleic acid which hybridizes under wash conditions of 2 x SSC at 22°C, and more preferably 0.2 x SSC at 65°C, to a nucleotide represented by SEQ ID No. 1 or 3. Accordingly, the method may comprise introducing a dsRNA contruct into a cell in which Dicer has been recombinantly expressed or otherwise ectopically activated.

In certain embodiment, at least one of the activated RNAi enzymes is Argonaut, or a homolog thereof. In certain preferred embodiments, the present method provides for ectopic activation of Argonaut. As used herein, the term "Argonaut" refers to a protein which (a) mediates an RNAi response and (b) has an amino acid sequence at least 50 percent identical, and more preferably at least 75, 85, 90 or 95 percent identical to the amino acid sequence shown in Figure 24. Accordingly, the method may comprise introducing a dsRNA contruct into a cell in which Argonaut has been recombinantly expressed or otherwise ectopically activated.

This invention also provides expression vectors containing a nucleic acid encoding a Dicer or Argonaut polypeptide, operably linked to at least one transcriptional regulatory sequence. Operably linked is intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence. Regulatory sequences are art-recognized and are selected to direct expression of the subject Dicer or Argonaut proteins. Accordingly, the term transcriptional regulatory sequence includes promoters, enhancers and other expression control elements. Such

regulatory sequences are described in Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences, sequences that control the expression of a DNA sequence when operatively linked to it, may be used in these vectors to express DNA sequences encoding Dicer or Argonaut polypeptides of this invention. Such useful expression control sequences, include, for example, a viral LTR, such as the LTR of the Moloney murine leukemia virus, the early and late promoters of SV40, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage λ, the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed.

Moreover, the vector's copy number, the ability to control that copy number and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered.

The recombinant Dicer or Argonaut genes can be produced by ligating a nucleic acid encoding a Dicer or Argonaut polypeptide into a vector suitable for expression in either prokaryotic cells, eukaryotic cells, or both. Expression vectors for production of recombinant forms of the subject Dicer or Argonaut polypeptides include plasmids and other vectors. For instance, suitable vectors for the expression of a Dicer or Argonaut polypeptide include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as E. coli.

A number of vectors exist for the expression of recombinant proteins in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression vehicles useful in the introduction of genetic constructs into S. cerevisiae (see, for example, Broach et al. (1983) in Experimental Manipulation of Gene Expression, ed. M. Inouye Academic Press, p. 83, incorporated by reference herein). These vectors can replicate in E. coli due the presence of the pBR322 ori, and in S. cerevisiae due to the replication determinant of the yeast 2 micron plasmid. In addition, drug resistance markers such as ampicillin can be used. In an illustrative embodiment, a Dicer or

Argonaut polypeptide is produced recombinantly utilizing an expression vector generated by sub-cloning the coding sequence of a Dicer or Argonaut gene.

The preferred mammalian expression vectors contain both prokaryotic sequences, to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papillomavirus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989) Chapters 16 and 17.

In yet another embodiment, the subject invention provides a "gene activation" construct which, by homologous recombination with a genomic DNA, alters the transcriptional regulatory sequences of an endogenous Dicer or Argonaut gene. For instance, the gene activation construct can replace the endogenous promoter of a Dicer or Argonaut gene with a heterologous promoter, e.g., one which causes constitutive expression of the Dicer or Argonaut gene or which causes inducible expression of the gene under conditions different from the normal expression pattern of Dicer or Argonaut. A variety of different formats for the gene activation constructs are available. See, for example, the Transkaryotic Therapies, Inc PCT publications WO93/09222, WO95/31560, WO96/29411, WO95/31560 and WO94/12650.

In preferred embodiments, the nucleotide sequence used as the gene activation construct can be comprised of (1) DNA from some portion of the endogenous Dicer or Argonaut gene (exon sequence, intron sequence, promoter sequences, etc.) which direct recombination and (2) heterologous transcriptional regulatory sequence(s) which is to be operably linked to the coding sequence for the genomic Dicer or Argonaut gene upon recombination of the gene activation construct. For use in generating cultures of Dicer or Argonaut producing cells, the construct may further include a reporter gene to detect the presence of the knockout construct in the cell.

The gene activation construct is inserted into a cell, and integrates with the genomic DNA of the cell in such a position so as to provide the heterologous regulatory

sequences in operative association with the native Dicer or Argonaut gene. Such insertion occurs by homologous recombination, i.e., recombination regions of the activation construct that are homologous to the endogenous Dicer or Argonaut gene sequence hybridize to the genomic DNA and recombine with the genomic sequences so that the construct is incorporated into the corresponding position of the genomic DNA.

The terms "recombination region" or "targeting sequence" refer to a segment (i.e., a portion) of a gene activation construct having a sequence that is substantially identical to or substantially complementary to a genomic gene sequence, e.g., including 5' flanking sequences of the genomic gene, and can facilitate homologous recombination between the genomic sequence and the targeting transgene construct.

As used herein, the term "replacement region" refers to a portion of a activation construct which becomes integrated into an endogenous chromosomal location following homologous recombination between a recombination region and a genomic sequence.

The heterologous regulatory sequences, e.g., which are provided in the replacement region, can include one or more of a variety of elements, including: promoters (such as constitutive or inducible promoters), enhancers, negative regulatory elements, locus control regions, transcription factor binding sites, or combinations thereof.

Promoters/enhancers which may be used to control the expression of the targeted gene *in vivo* include, but are not limited to, the cytomegalovirus (CMV) promoter/enhancer (Karasuyama et al. (1989) J. Exp. Med 169: 13), the human β-actin promoter (Gunning et al. (1987) PNAS 84: 4831-4835), the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR) (Klessig et al. (1984) Mol. Cell Biol. 4: 1354-1362), the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR) (Weiss et al. (1985) RNA Tumor Viruses, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), the SV40 early or late region promoter (Bernoist et al. (1981) *Nature* 290: 304-310; Templeton et al. (1984) Mol. Cell Biol. 4: 817; and Sprague et al. (1983) J. Virol. 45: 773), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (RSV) (Yamamoto et al. (1980) Cell 22: 787-797), the herpes simplex virus (HSV) thymidine kinase promoter/enhancer (Wagner et al. (1981) PNAS 82: 3567-71), and the herpes simplex virus LAT promoter (Wolfe et al. (1992) Nature Genetics 1: 379-384).

In still other embodiments, the replacement region merely deletes a negative transcriptional control element of the native gene, e.g., to activate expression, or ablates a positive control element, e.g., to inhibit expression of the targeted gene.

B. Cell/Organism

The cell with the target gene may be derived from or contained in any organism (e.g., plant, animal, protozoan, virus, bacterium, or fungus). The dsRNA construct may be synthesized either in vivo or in vitro. Endogenous RNA polymerase of the cell may mediate transcription in vivo, or cloned RNA polymerase can be used for transcription in vivo or in vitro. For generating double stranded transcripts from a transgene in vivo, a regulatory region may be used to transcribe the RNA strand (or strands). Furthermore, dsRNA can be generated by transcribing an RNA strand which forms a hairpin, thus producing a dsRNA.

Genetic manipulation becomes possible in organisms that are not classical genetic models. Breeding and screening programs may be accelerated by the ability to rapidly assay the consequences of a specific, targeted gene disruption. Gene disruptions may be used to discover the function of the target gene, to produce disease models in which the target gene are involved in causing or preventing a pathological condition, and to produce organisms with improved economic properties.

The cell with the target gene may be derived from or contained in any organism. The organism may be a plant, animal, protozoan, bacterium, virus, or fungus. The plant may be a monocot, dicot or gymnosperm; the animal may be a vertebrate or invertebrate. Preferred microbes are those used in agriculture or by industry, and those that are pathogenic for plants or animals. Fungi include organisms in both the mold and yeast morphologies.

Plants include arabidopsis; field crops (e.g., alfalfa, barley, bean, com, cotton, flax, pea, rape, rice, rye, safflower, sorghum, soybean, sunflower, tobacco, and wheat); vegetable crops (e.g., asparagus, beet, broccoli, cabbage, carrot, cauliflower, celery, cucumber, eggplant, lettuce, onion, pepper, potato, pumpkin, radish, spinach, squash, taro, tomato, and zucchini); fruit and nut crops (e.g., almond, apple, apricot, banana, blackberry, blueberry, cacao, cherry, coconut, cranberry, date, faJoa, filbert, grape, grapefruit, guava, kiwi, lemon, lime, mango, melon, nectarine, orange, papaya, passion fruit, peach, peanut, pear, pineapple, pistachio, plum, raspberry, strawberry, tangerine, walnut, and watermelon); and ornamentals (e.g., alder, ash, aspen, azalea, birch, boxwood, camellia, carnation, chrysanthemum, elm, fir, ivy, jasmine, juniper, oak, palm, poplar, pine, redwood, rhododendron, rose, and rubber).

Examples of vertebrate animals include fish, mammal, cattle, goat, pig, sheep, rodent, hamster, mouse, rat, primate, and human.

Invertebrate animals include nematodes, other worms, drosophila, and other insects. Representative generae of nematodes include those that infect animals (e.g., Ancylostoma, Ascaridia, Ascaris, Bunostomum, Caenorhabditis, Capillaria, Chabertia, Cooperia, Dictyocaulus, Haernonchus, Heterakis, Nematodirus, Oesophagostomum,

Ostertagia, Oxyuris, Parascaris, Strongylus, Toxascaris, Trichuris, Trichostrongylus, Tflichonema, Toxocara, Uncinaria) and those that infect plants (e.g., Bursaphalenchus, Criconerriella, Diiylenchus, Ditylenchus, Globodera, Helicotylenchus, Heterodera, Longidorus, Melodoigyne, Nacobbus, Paratylenchus, Pratylenchus, Radopholus, Rotelynchus, Tylenchus, and Xiphinerna). Representative orders of insects include Coleoptera, Diptera, Lepidoptera, and Homoptera.

The cell having the target gene may be from the germ line or somatic, totipotent or pluripotent, dividing or non-dividing, parenchyma or epithelium, immortalized or transformed, or the like. The cell may be a stem cell or a differentiated cell. Cell types that are differentiated include adipocytes, fibroblasts, myocytes, cardiomyocytes, endothelium, neurons, glia, blood cells, megakaryocytes, lymphocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, leukocytes, granulocytes, keratinocytes, chondrocytes, osteoblasts, osteoclasts, hepatocytes, and cells of the endocrine or exocrine glands.

C. Targeted Genes

The target gene may be a gene derived from the cell, an endogenous gene, a transgene, or a gene of a pathogen which is present in the cell after infection thereof. Depending on the particular target gene and the dose of double stranded RNA material delivered, the procedure may provide partial or complete loss of function for the target gene. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells. Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein.

"Inhibition of gene expression" refers to the absence (or observable decrease) in the level of protein and/or mRNA product from a target gene. "Specificity" refers to the ability to inhibit the target gene without manifest effects on other genes of the cell. The consequences of inhibition can be confirmed by examination of the outward properties of the cell or organism (as presented below in the examples) or by biochemical techniques such as RNA solution hybridization, nuclease protection, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme linked immunosorbent assay (ELISA), Western blotting, radioimmunoassay (RIA), other immunoassays, and fluorescence activated cell analysis (FACS). For RNA-mediated inhibition in a cell line or whole organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Such reporter genes include acetohydroxyacid synthase (AHAS), alkaline phosphatase (AP), beta galactosidase (LacZ), beta glucoronidase (GUS), chloramphenicol acetyltransferase

(CAT), green fluorescent protein (GFP), horseradish peroxidase (HRP), luciferase (Luc), nopaline synthase (NOS), octopine synthase (OCS), and derivatives thereof. Multiple selectable markers are available that confer resistance to ampicillin, bleomycin, chloramphenicol, gentamycin, hygromycin, kanamycin, lincomycin, methotrexate, phosphinothricin, puromycin, and tetracyclin.

Depending on the assay, quantitation of the amount of gene expression allows one to determine a degree of inhibition which is greater than 10%, 33%, 50%, 90%, 95% or 99% as compared to a cell not treated according to the present invention. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells (e.g., at least 10%, 20%, 50%, 75%, 90%, or 95% of targeted cells). Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein. As an example, the efficiency of inhibition may be determined by assessing the amount of gene product in the cell: mRNA may be detected with a hybridization probe having a nucleotide sequence outside the region used for the inhibitory double-stranded RNA, or translated polypeptide may be detected with an antibody raised against the polypeptide sequence of that region.

As disclosed herein, the present invention is not limited to any type of target gene or nucleotide sequence. But the following classes of possible target genes are listed for illustrative purposes: developmental genes (e.g., adhesion molecules, cyclin kinase inhibitors, Writ family members, Pax family members, Winged helix family members, Hox family members, cytokines/lymphokines and their receptors, growth/differentiation factors and their receptors, neurotransmitters and their receptors); oncogenes (e.g., ABLI, BCLI, BCL2, BCL6, CBFA2, CBL, CSFIR, ERBA, ERBB, EBRB2, ETSI, ETS1, ETV6, FGR, FOS, FYN, HCR, HRAS, JUN, KRAS, LCK, LYN, MDM2, MLL, MYB, MYC, MYCLI, MYCN, NRAS, PIM 1, PML, RET, SRC, TALI, TCL3, and YES); tumor suppressor genes (e.g., APC, BRCA 1, BRCA2, MADH4, MCC, NF 1, NF2, RB 1, TP53, and WTI); and enzymes (e.g., ACC synthases and oxidases, ACP desaturases and hydroxylases, ADP-glucose pyrophorylases, ATPases, alcohol dehydrogenases, amylases, amyloglucosidases, catalases, cellulases, chalcone synthases, chitinases, cyclooxygenases, decarboxylases, dextrinases, DNA and RNA polymerases, galactosidases, glucanases, glucose oxidases, granule-bound starch synthases, GTPases, helicases, hemicellulases, integrases, inulinases, invertases, isomerases, kinases, lactases, lipases, lipoxygenases, lysozymes, nopaline synthases, octopine synthases, pectinesterases, peroxidases, phosphatases, phospholipases, phosphorylases, phytases, plant growth regulator synthases, polygalacturonases, proteinases and peptidases, pullanases, recombinases, reverse transcriptases, RUBISCOs, topoisomerases, and xylanases).

D. dsRNA constructs

The dsRNA construct may comprise one or more strands of polymerized ribonucleotide. It may include modifications to either the phosphate-sugar backbone or the nucleoside. For example, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. Modifications in RNA structure may be tailored to allow specific genetic inhibition while avoiding a general panic response in some organisms which is generated by dsRNA. Likewise, bases may be modified to block the activity of adenosine deaminase. The dsRNA construct may be produced enzymatically or by partial/total organic synthesis, any modified ribonucieotide can be introduced by in vitro enzymatic or organic synthesis.

The dsRNA construct may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing an organism in a solution containing RNA. Methods for oral introduction include direct mixing of RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express an RNA, then fed to the organism to be affected. Physical methods of introducing nucleic acids include injection of an RNA solution directly into the cell or extracellular injection into the organism.

The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses (e.g., at least 5, 10, 100, 500 or 1000 copies per cell) of double-stranded material may yield more effective inhibition; lower doses may also be useful for specific applications. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition.

dsRNA constructs containing a nucleotide sequences identical to a portion, of either coding or non-coding sequence, of the target gene are preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence (ds RNA similar to the target gene) have also been found to be effective for inhibition. Thus, sequence identity may be optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, Sequence Analysis Primer, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (e.g., University of Wisconsin Genetic Computing Group). Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and the portion of

the target gene is preferred. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript (e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50 °C or 70 °C hybridization for 12-16 hours; followed by washing). In certain preferred embodiments, the length of the dsRNA is at least 20, 21 or 22 nucleotides in length, e.g., corresponding in size to RNA products produced by Dicer-dependent cleavage. In certain embodiments, the dsRNA construct is at least 25, 50, 100, 200, 300 or 400 bases. In certain embodiments, the dsRNA construct is 400-800 bases in length.

100% sequence identity between the RNA and the target gene is not required to practice the present invention. Thus the invention has the advantage of being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence.

The dsRNA construct may be synthesized either in vivo or in vitro. Endogenous RNA polymerase of the cell may mediate transcription in vivo, or cloned RNA polymerase can be used for transcription in vivo or in vitro. For transcription from a transgene in vivo or an expression construct, a regulatory region (e.g., promoter, enhancer, silencer, splice donor and acceptor, polyadenylation) may be used to transcribe the dsRNA strand (or strands). Inhibition may be targeted by specific transcription in an organ, tissue, or cell type; stimulation of an environmental condition (e.g., infection, stress, temperature, chemical inducers); and/or engineering transcription at a developmental stage or age. The RNA strands may or may not be polyadenylated; the RNA strands may or may not be capable of being translated into a polypeptide by a cell's translational apparatus. The dsRNA construct may be chemically or enzymatically synthesized by manual or automated reactions. The dsRNA construct may be synthesized by a cellular RNA polymerase or a bacteriophage RNA polymerase (e.g., T3, T7, SP6). The use and production of an expression construct are known in the art (see also WO 97/32016; U.S. Pat. Nos. 5,593,874, 5,698,425, 5,712,135, 5,789,214, and 5,804,693; and the references cited therein). If synthesized chemically or by in vitro enzymatic synthesis, the RNA may be purified prior to introduction into the cell. For example, RNA can be purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography or a combination thereof. Alternatively, the dsRNA construct may be used with no or a minimum of purification to avoid losses due to sample processing. The dsRNA construct may be dried for storage or dissolved in an aqueous solution. The solution may contain buffers or salts to promote annealing, and/or stabilization of the duplex strands.

Physical methods of introducing nucleic acids include injection of a solution containing the dsRNA construct, bombardment by particles covered by the dsRNA

construct, soaking the cell or organism in a solution of the RNA, or electroporation of cell membranes in the presence of the dsRNA construct. A viral construct packaged into a viral particle would accomplish both efficient introduction of an expression construct into the cell and transcription of dsRNA construct encoded by the expression construct. Other methods known in the art for introducing nucleic acids to cells may be used, such as lipid-mediated carrier transport, chemical mediated transport, such as calcium phosphate, and the like. Thus the dsRNA construct may be introduced along with components that perform one or more of the following activities: enhance RNA uptake by the cell, promote annealing of the duplex strands, stabilize the annealed strands, or other-wise increase inhibition of the target gene.

E. Illustrative Uses

One utility of the present invention is as a method of identifying gene function in an organism, especially higher eukaryotes, comprising the use of double-stranded RNA to inhibit the activity of a target gene of previously unknown function. Instead of the time consuming and laborious isolation of mutants by traditional genetic screening, functional genomics would envision determining the function of uncharacterized genes by employing the invention to reduce the amount and/or alter the timing of target gene activity. The invention could be used in determining potential targets for pharmaceuticals, understanding normal and pathological events associated with development, determining signaling pathways responsible for postnatal development/aging, and the like. The increasing speed of acquiring nucleotide sequence information from genomic and expressed gene sources, including total sequences for mammalian genomes, can be coupled with the invention to determine gene function in a cell or in a whole organism. The preference of different organisms to use particular codons, searching sequence databases for related gene products, correlating the linkage map of genetic traits with the physical map from which the nucleotide sequences are derived, and artificial intelligence methods may be used to define putative open reading frames from the nucleotide sequences acquired in such sequencing projects.

A simple assay would be to inhibit gene expression according to the partial sequence available from an expressed sequence tag (EST). Functional alterations in growth, development, metabolism, disease resistance, or other biological processes would be indicative of the normal role of the EST's gene product.

The ease with which the dsRNA construct can be introduced into an intact cell/organism containing the target gene allows the present invention to be used in high throughput screening (HTS). For example, duplex RNA can be produced by an amplification reaction using primers flanking the inserts of any gene library derived from

the target cell or organism. Inserts may be derived from genomic DNA or mRNA (e.g., cDNA and cRNA). Individual clones from the library can be replicated and then isolated in separate reactions, but preferably the library is maintained in individual reaction vessels (e.g., a 96 well microtiter plate) to minimize the number of steps required to practice the invention and to allow automation of the process.

In an exemplary embodiment, the subject invention provides an arrayed library of RNAi constructs. The array may be in the form of solutions, such as multi-well plates, or may be "printed" on solid substrates upon which cells can be grown. To illustrate, solutions containing duplex RNAs that are capable of inhibiting the different expressed genes can be placed into individual wells positioned on a microtiter plate as an ordered array, and intact cells/organisms in each well can be assayed for any changes or modifications in behavior or development due to inhibition of target gene activity.

In one embodiment, the subject method uses an arrayed library of RNAi constructs to screen for combinations of RNAi that are lethal to host cells. Synthetic lethality is a bedrock principle of experimental genetics. A synthetic lethality describes the properties of two mutations which, individually, are tolerated by the organism but which, in combination, are lethal. The subject arrays can be used to identify loss-of-function mutations that are lethal in combination with alterations in other genes, such as activated oncogenes or loss-of-function mutations to tumor suppressors. To achieve this, one can create "phenotype arrays" using cultured cells. Expression of each of a set of genes, such as the host cell's genome, can be individually systematically disrupted using RNA interference. Combination with alterations in oncogene and tumor suppressor pathways can be used to identify synthetic lethal interactions that may identify novel therapeutic targets.

In certain embodiments, the RNAi constructs can be fed directly to, or injected into, the cell/organism containing the target gene. Alternatively, the duplex RNA can be produced by in vivo or in vitro transcription from an expression construct used to produce the library. The construct can be replicated as individual clones of the library and transcribed to produce the RNA; each clone can then be fed to, injected into, or delivered by another method known in the art to,the cell/organism containing the target gene. The function of the target gene can be assayed from the effects it has on the cell/organism when gene activity is inhibited. This screening could be amenable to small subjects that can be processed in large number, for example, tissue culture cells derived from mammals, especially primates, and most preferably humans.

If a characteristic of an organism is determined to be genetically linked to a polymorphism through RFLP or QTL analysis, the present invention can be used to gain insight regarding whether that genetic polymorphism might be directly responsible for the

characteristic. For example, a fragment defining the genetic polymorphism or sequences in the vicinity of such a genetic polymorphism can be amplified to produce an RNA, the duplex RNA can be introduced to the organism or cell, and whether an alteration in the characteristic is correlated with inhibition can be determined. Of course, there may be trivial explanations for negative results with this type of assay, for example: inhibition of the target gene causes lethality, inhibition of the target gene may not result in any observable alteration, the fragment contains nucleotide sequences that are not capable of inhibiting the target gene, or the target gene's activity is redundant.

The present invention may be useful in allowing the inhibition of essential genes. Such genes may be required for cell or organism viability at only particular stages of development or only in specific cellular compartments or tissues. The functional equivalent of conditional mutations may be produced by inhibiting activity of the target gene when or where it is not required for viability. The invention allows addition of RNA at specific times of development and locations in the organism without introducing permanent mutations into the target genome.

The present invention may be useful in allowing the inhibition of genes that have been difficult to inhibit using other methods due to gene redundancy. Since the present methods may be used to deliver more than one dsRNA to a cell or organism, dsRNA identical or similar to more than one gene, wherein the genes have a redundant function during normal development, may be delivered.

If alternative splicing produced a family of transcripts that were distinguished by usage of characteristic exons, the present invention can target inhibition through the appropriate exons to specifically inhibit or to distinguish among the functions of family members. For example, a protein factor that contained an alternatively spliced transmembrane domain may be expressed in both membrane bound and secreted forms. Instead of isolating a nonsense mutation that terminates translation before the transmembrane domain, the functional consequences of having only secreted hormone can be determined according to the invention by targeting the exon containing the transmembrane domain and thereby inhibiting expression of membrane-bound hormone. That is, the subject method can be used for selected ablation of splicing variants.

The present invention may be used alone or as a component of a kit having at least one of the reagents necessary to carry out the in vitro or in vivo introduction of RNA to test samples or subjects. Preferred components are the dsRNA and a vehicle that promotes introduction of the dsRNA. Such a kit may also include instructions to allow a user of the kit to practice the invention.

Alternatively, an organism may be engineered to produce dsRNA which produces commercially or medically beneficial results, for example, resistance to a pathogen or its pathogenic effects, improved growth, or novel developmental patterns.

IV. Exemplification

The invention, now being generally described, will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention and are not intended to limit the invention.

Example 1: An RNA-Directed Nuclease Mediates RNAi Gene Silencing

In a diverse group of organisms that includes *Caenorhabditis elegans*, *Drosophila*, planaria, hydra, trypanosomes, fungi and plants, the introduction of double-stranded RNAs inhibits gene expression in a sequence-specific manner (Sharp (1999) Genes and Development 13: 139-141; Sanchez-Alvarado and Newmark (1999) PNAS 96: 5049-5054; Lohman et al. (1999) Developmental Biology 214: 211-214; Cogoni and Macino (1999) Nature 399: 166-169; Waterhouse et al. (1998) PNAS 95: 13959-13964; Montgomery and Fire (1998) Trends Genet. 14: 225-228; Ngo et al. (1998) PNAS 95: 14687-14692). These responses, called RNA interference or post-transcriptional gene silencing, may provide anti-viral defence, modulate transposition or regulate gene expression (Sharp (1999) Genes and Development 13: 139-141; Montgomery and Fire (1998) Trends Genet. 14: 225-228; Tabara et al. (1999) Cell 99: 123-132; Ketting et al. (1999) Cell 99: 133-141; Ratcliff et al. (1997) Science 276: 1558-1560). We have taken a biochemical approach towards elucidating the mechanisms underlying this genetic phenomenon. Here we show that 'loss-of-function' phenotypes can be created in cultured *Drosophila* cells by transfection with specific double-stranded RNAs. This coincides with a marked reduction in the level of cognate cellular messenger RNAs. Extracts of transfected cells contain a nuclease activity that specifically degrades exogenous transcripts homologous to transfected double-stranded RNA. This enzyme contains an essential RNA component. After partial purification, the sequence-specific nuclease co-fractionates with a discrete, ~25-nucleotide RNA species which may confer specificity to the enzyme through homology to the substrate mRNAs.

Although double-stranded RNAs (dsRNAs) can provoke gene silencing in numerous biological contexts including *Drosophila* (Kennerdell et al. (1998) Cell 95: 1017-1026; Misquitta and Paterson (1999) PNAS 96: 1451-1456), the mechanisms underlying this phenomenon have remained mostly unknown. We therefore wanted to

establish a biochemically tractable model in which such mechanisms could be investigated.

Transient transfection of cultured, *Drosophila* S2 cells with a *lacZ* expression vector resulted in β-galactosidase activity that was easily detectable by an *in situ* assay (Fig. 1a). This activity was greatly reduced by co-transfection with a dsRNA corresponding to the first 300 nucleotides of the *lacZ* sequence, whereas co-transfection with a control dsRNA (*CD8*) (Fig. 1a) or with single-stranded RNAs of either sense or antisense orientation (data not shown) had little or no effect. This indicated that dsRNAs could interfere, in a sequence-specific fashion, with gene expression in cultured cells.

To determine whether RNA interference (RNAi) could be used to target endogenous genes, we transfected S2 cells with a dsRNA corresponding to the first 540 nucleotides of *Drosophila cyclin E*, a gene that is essential for progression into S phase of the cell cycle. During log-phase growth, untreated S2 cells reside primarily in G2/M (Fig. 1b). Transfection with *lacZ* dsRNA had no effect on cell-cycle distribution, but transfection with the *cyclin E* dsRNA caused a G1-phase cell-cycle arrest (Fig. 1b). The ability of *cyclin E* dsRNA to provoke this response was length-dependent. Double-stranded RNAs of 540 and 400 nucleotides were quite effective, whereas dsRNAs of 200 and 300 nucleotides were less potent. Double-stranded *cyclin E* RNAs of 50 or 100 nucleotides were inert in our assay, and transfection with a single-stranded, antisense *cyclin E* RNA had virtually no effect.

One hallmark of RNAi is a reduction in the level of mRNAs that are homologous to the dsRNA. Cells transfected with the *cyclin E* dsRNA (bulk population) showed diminished endogenous *cyclin E* mRNA as compared with control cells (Fig. 1c). Similarly, transfection of cells with dsRNAs homologous to *fizzy*, a component of the anaphase-promoting complex (APC) or *cyclin A*, a cyclin that acts in S, G2 and M, also caused reduction of their cognate mRNAs (Fig.1c). The modest reduction in *fizzy* mRNA levels in cells transfected with *cyclin A* dsRNA probably resulted from arrest at a point in the division cycle at which *fizzy* transcription is low (Wolf and Jackson (1998) Current Biology 8: R637-R639; Kramer et al. (1998) Current Biology 8: 1207-1210). These results indicate that RNAi may be a generally applicable method for probing gene function in cultured *Drosophila* cells.

The decrease in mRNA levels observed upon transfection of specific dsRNAs into *Drosophila* cells could be explained by effects at transcriptional or post-transcriptional levels. Data from other systems have indicated that some elements of the dsRNA response may affect mRNA directly (reviewed in Sharp (1999) Genes and Development 13: 139-141; Montgomery and Fire (1998) Trends Genet. 14: 225-228). We therefore sought to develop a cell-free assay that reflected, at least in part, RNAi.

S2 cells were transfected with dsRNAs corresponding to either *cyclin E* or *lacZ*. Cellular extracts were incubated with synthetic mRNAs of *lacZ* or *cyclin E*. Extracts prepared from cells transfected with the 540-nucleotide *cyclin E* dsRNA efficiently degraded the *cyclin E* transcript; however, the *lacZ* transcript was stable in these lysates (Fig. 2a). Conversely, lysates from cells transfected with the *lacZ* dsRNA degraded the *lacZ* transcript but left the *cyclin E* mRNA intact. These results indicate that RNAi ablates target mRNAs through the generation of a sequence-specific nuclease activity. We have termed this enzyme RISC (RNA-induced silencing complex). Although we occasionally observed possible intermediates in the degradation process (see Fig. 2), the absence of stable cleavage end-products indicates an exonuclease (perhaps coupled to an endonuclease). However, it is possible that the RNAi nuclease makes an initial endonucleolytic cut and that non-specific exonucleases in the extract complete the degradation process (Shuttleworth and Colman (1988) EMBO J. 7: 427-434). In addition, our ability to create an extract that targets *lacZ in vitro* indicates that the presence of an endogenous gene is not required for the RNAi response.

To examine the substrate requirements for the dsRNA-induced, sequence-specific nuclease activity, we incubated a variety of *cyclin-E*-derived transcripts with an extract derived from cells that had been transfected with the 540-nucleotide *cyclin E* dsRNA (Fig. 2b, c). Just as a length requirement was observed for the transfected dsRNA, the RNAi nuclease activity showed a dependence on the size of the RNA substrate. Both a 600-nucleotide transcript that extends slightly beyond the targeted region (Fig. 2b) and an ~1-kilobase (kb) transcript that contains the entire coding sequence (data not shown) were completely destroyed by the extract. Surprisingly, shorter substrates were not degraded as efficiently. Reduced activity was observed against either a 300- or a 220-nucleotide transcript, and a 100-nucleotide transcript was resistant to nuclease in our assay. This was not due solely to position effects because ~100-nucleotide transcripts derived from other portions of the transfected dsRNA behaved similarly (data not shown). As expected, the nuclease activity (or activities) present in the extract could also recognize the antisense strand of the *cyclin E* mRNA. Again, substrates that contained a substantial portion of the targeted region were degraded efficiently whereas those that contained a shorter stretch of homologous sequence (~130 nucleotides) were recognized inefficiently (Fig. 2c, as600). For both the sense and antisense strands, transcripts that had no homology with the transfected dsRNA (Fig. 2b, Eout; Fig. 2c, as300) were not degraded. Although we cannot exclude the possibility that nuclease specificity could have migrated beyond the targeted region, the resistance of transcripts that do not contain homology to the dsRNA is consistent with data from *C. elegans*. Double-stranded RNAs homologous to an upstream cistron have little or no effect on a linked downstream cistron, despite the fact that unprocessed, polycistronic mRNAs can be readily detected (Tabara et al. (1998) Science

282: 430-432; Bosher et al. (1999) Genetics 153: 1245-1256). Furthermore, the nuclease was inactive against a dsRNA identical to that used to provoke the RNAi response *in vivo* (Fig. 2b). In the *in vitro* system, neither a 5' cap nor a poly(A) tail was required, as such transcripts were degraded as efficiently as uncapped and non-polyadenylated RNAs.

Gene silencing provoked by dsRNA is sequence specific. A plausible mechanism for determining specificity would be incorporation of nucleic-acid guide sequences into the complexes that accomplish silencing (Hamilton and Baulcombe (1999) Science 286: 950-952). In accord with this idea, pre-treatment of extracts with a Ca^{2+}-dependent nuclease (micrococcal nuclease) abolished the ability of these extracts to degrade cognate mRNAs (Fig. 3). Activity could not be rescued by addition of non-specific RNAs such as yeast transfer RNA. Although micrococcal nuclease can degrade both DNA and RNA, treatment of the extract with DNAse I had no effect (Fig. 3). Sequence-specific nuclease activity, however, did require protein (data not shown). Together, our results support the possibility that the RNAi nuclease is a ribonucleoprotein, requiring both RNA and protein components. Biochemical fractionation (see below) is consistent with these components being associated in extract rather than being assembled on the target mRNA after its addition.

In plants, the phenomenon of co-suppression has been associated with the existence of small (~25-nucleotide) RNAs that correspond to the gene that is being silenced (Hamilton and Baulcombe (1999) Science 286: 950-952). To address the possibility that a similar RNA might exist in *Drosophila* and guide the sequence-specific nuclease in the choice of substrate, we partially purified our activity through several fractionation steps. Crude extracts contained both sequence-specific nuclease activity and abundant, heterogeneous RNAs homologous to the transfected dsRNA (Figs 2 and 4a). The RNAi nuclease fractionated with ribosomes in a high-speed centrifugation step. Activity could be extracted by treatment with high salt, and ribosomes could be removed by an additional centrifugation step. Chromatography of soluble nuclease over an anion-exchange column resulted in a discrete peak of activity (Fig. 4b, *cyclin E*). This retained specificity as it was inactive against a heterologous mRNA (Fig. 4b, *lacZ*). Active fractions also contained an RNA species of 25 nucleotides that is homologous to the *cyclin E* target (Fig. 4b, northern). The band observed on northern blots may represent a family of discrete RNAs because it could be detected with probes specific for both the sense and antisense *cyclin E* sequences and with probes derived from distinct segments of the dsRNA (data not shown). At present, we cannot determine whether the 25-nucleotide RNA is present in the nuclease complex in a double-stranded or single-stranded form.

RNA interference allows an adaptive defence against both exogenous and endogenous dsRNAs, providing something akin to a dsRNA immune response. Our data,

and that of others (Hamilton and Baulcombe (1999) Science 286: 950-952), is consistent with a model in which dsRNAs present in a cell are converted, either through processing or replication, into small specificity determinants of discrete size in a manner analogous to antigen processing. Our results suggest that the post-transcriptional component of dsRNA-dependent gene silencing is accomplished by a sequence-specific nuclease that incorporates these small RNAs as guides that target specific messages based upon sequence recognition. The identical size of putative specificity determinants in plants (Hamilton and Baulcombe (1999) Science 286: 950-952) and animals predicts a conservation of both the mechanisms and the components of dsRNA-induced, post-transcriptional gene silencing in diverse organisms. In plants, dsRNAs provoke not only post-transcriptional gene silencing but also chromatin remodelling and transcriptional repression (Jones et al. (1998) EMBO J. 17: 6385-6393; Jones et al. (1999) Plant Cell 11: 2291-2301). It is now critical to determine whether conservation of gene-silencing mechanisms also exists at the transcriptional level and whether chromatin remodelling can be directed in a sequence-specific fashion by these same dsRNA-derived guide sequences.

Methods

Cell culture and RNA methods S2 cells (Schneider (1972) J. Embryol Exp Morpho 27: 353-365) were cultured at 27 °C in 90% Schneider's insect media (Sigma), 10% heat inactivated fetal bovine serum (FBS). Cells were transfected with dsRNA and plasmid DNA by calcium phosphate co-precipitation (DiNocera and Dawid (1983) PNAS 80: 7095-7098). Identical results were observed when cells were transfected using lipid reagents (for example, Superfect, Qiagen). For FACS analysis, cells were additionally transfected with a vector that directs expression of a green fluorescent protein (GFP)–US9 fusion protein (Kalejta et al. (1999) Exp Cell Res. 248: 322-328). These cells were fixed in 90% ice-cold ethanol and stained with propidium iodide at 25 μg/ml. FACS was performed on an Elite flow cytometer (Coulter). For northern blotting, equal loading was ensured by over-probing blots with a control complementary DNA (RP49). For the production of dsRNA, transcription templates were generated by polymerase chain reaction such that they contained T7 promoter sequences on each end of the template. RNA was prepared using the RiboMax kit (Promega). Confirmation that RNAs were double stranded came from their complete sensitivity to RNAse III (a gift from A. Nicholson). Target mRNA transcripts were synthesized using the Riboprobe kit (Promega) and were gel purified before use.

Extract preparation Log-phase S2 cells were plated on 15-cm tissue culture dishes and transfected with 30 μg dsRNA and 30 μg carrier plasmid DNA. Seventy-two hours after transfection, cells were harvested in PBS containing 5 mM EGTA, washed twice in PBS

and once in hypotonic buffer (10 mM HEPES pH 7.3, 6 mM β-mercaptoethanol). Cells were suspended in 0.7 packed-cell volumes of hypotonic buffer containing *Complete* protease inhibitors (Boehringer) and 0.5 units/ml of RNasin (Promega). Cells were disrupted in a dounce homogenizer with a type B pestle, and lysates were centrifuged at 30,000*g* for 20 min. Supernatants were used in an *in vitro* assay containing 20 mM HEPES pH 7.3, 110 mM KOAc, 1 mM $Mg(OAc)_2$, 3 mM EGTA, 2 mM $CaCl_2$, 1 mM DTT. Typically, 5 µl extract was used in a 10 µl assay that contained also 10,000 c.p.m. synthetic mRNA substrate.

Extract fractionation Extracts were centrifuged at 200,000*g* for 3 h and the resulting pellet (containing ribosomes) was extracted in hypotonic buffer containing also 1 mM $MgCl_2$ and 300 mM KOAc. The extracted material was spun at 100,000*g* for 1 h and the resulting supernatant was fractionated on Source 15Q column (Pharmacia) using a KCl gradient in buffer A (20 mM HEPES pH 7.0, 1 mM dithiothreitol, 1 mM $MgCl_2$). Fractions were assayed for nuclease activity as described above. For northern blotting, fractions were proteinase K/SDS treated, phenol extracted, and resolved on 15% acrylamide 8M urea gels. RNA was electroblotted onto Hybond N+ and probed with strand-specific riboprobes derived from cyclin E mRNA. Hybridization was carried out in 500 mM $NaPO_4$ pH 7.0, 15% formamide, 7% SDS, 1% BSA. Blots were washed in 1X SSC at 37–45 °C.

Example 2: Role for a Bidentate Ribonuclease in the Initiation Step of RNA Interference

Genetic approaches in worms, fungi and plants have identified a group of proteins that are essential for double-stranded RNA-induced gene silencing. Among these are ARGONAUTE family members (e.g. RDE1, QDE2) (Tabara et al. (1999) Cell 99: 123-132; Catalanotto et al. (2000) Nature 404: 245; Fagard et al. (2000) PNAS 97: 11650-11654), recQ-family helicases (MUT-7, QDE3) (Ketting et al. (1999) Cell 99: 133-141; Cogoni and Macino. (1999) Science 286: 2342-2344), and RNA-dependent RNA polymerases (e.g. EGO-1, QDE1, SGS2/SDE1) (Cogoni and Macino (1999) Nature 399: 166-169; Smardon et al. (2000) Current Biology 10: 169-178; Mourrain et al. (2000) Cell 101: 533-542; Dalmay et al. (2000) Cell 101: 543-553). While potential roles have been proposed, none of these genes has been assigned a definitive function in the silencing process. Biochemical studies have suggested that PTGS is accomplished by a multicomponent nuclease that targets mRNAs for degradation (Hammond et al. (2000) Nature 404: 293-296; Zamore et al. (2000) Cell 101 25-33; Tuschl et al. (1999) Genes and Development 13: 3191-3197). We have shown that the specificity of this complex may

derive from the incorporation of a small guide sequence that is homologous to the mRNA substrate (Hammond et al. (2000) Nature 404: 293-296). Originally identified in plants that were actively silencing transgenes (Hamilton and Baulcombe (1999) Science 286: 950-952), these ~22 nt. RNAs have been produced during RNAi *in vitro* using an extract prepared from *Drosophila* embryos (Zamore et al. (2000) Cell 101 25-33). Putative guide RNAs can also be produced in extracts from *Drosophila* S2 cells (Fig. 5a). With the goal of understanding the mechanism of post-transcriptional gene silencing, we have undertaken both biochemical fractionation and candidate gene approaches to identify the enzymes that execute each step of RNAi.

Our previous studies resulted in the partial purification of a nuclease, RISC, that is an effector of RNA interference. See Example 1. This enzyme was isolated from *Drosophila* S2 cells in which RNAi had been initiated *in vivo* by transfection with dsRNA. We first sought to determine whether the RISC enzyme and the enzyme that initiates RNAi via processing of dsRNA into 22mers are distinct activities. RISC activity could be largely cleared from extracts by high-speed centrifugation (100,000xg for 60 min.) while the activity that produces 22mers remained in the supernatant (Fig. 5b,c). This simple fractionation indicated that RISC and the 22mer-generating activity are separable and thus distinct enzymes. However, it seems likely that they might interact at some point during the silencing process.

RNAse III family members are among the few nucleases that show specificity for double-stranded RNA (Nicholson (1999) FEMS Microbiol Rev 23: 371-390). Analysis of the *Drosophila* and *C. elegans* genomes reveals several types of RNAse III enzymes. First is the canonical RNAse III which contains a single RNAse III signature motif and a double-stranded RNA binding domain (dsRBD; e.g. RNC_CAEEL). Second is a class represented by Drosha (Filippov et al. (2000) Gene 245: 213-221), a *Drosophila* enzyme that contains two RNAse III motifs and a dsRBD (CeDrosha in *C. elegans*). A third class contains two RNAse III signatures and an amino terminal helicase domain (e.g. *Drosophila* CG4792, CG6493, *C. elegans* K12H4.8), and these had previously been proposed by Bass as candidate RNAi nucleases (Bass (2000) Cell 101: 235-238). Representatives of all three classes were tested for the ability to produce discrete, ~22 nt. RNAs from dsRNA substrates.

Partial digestion of a 500 nt. cyclin E dsRNA with purified, bacterial RNAse III produced a smear of products while nearly complete digestion produced a heterogeneous group of ~11-17 nucleotide RNAs (not shown). In order to test the dual-RNAse III enzymes, we prepared T7 epitope-tagged versions of Drosha and CG4792. These were expressed in transfected S2 cells and isolated by immunoprecipitation using antibody-agarose conjugates. Treatment of the dsRNA with the CG4792 immunoprecipitate yielded

~22 nt. fragments similar to those produced in either S2 or embryo extracts (Fig. 6a). Neither activity in extract nor activity in immunoprecipitates depended on the sequence of the RNA substrate since dsRNAs derived from several genes were processed equivalently (see Supplement 1). Negative results were obtained with Drosha and with immunoprecipitates of a DExH box helicase (Homeless (Gillespie et al. (1995) Genes and Development 9: 2495-2508); see Fig 6a,b). Western blotting confirmed that each of the tagged proteins was expressed and immunoprecipitated similarly (see Supplement 2). Thus, we conclude that CG4792 may carry out the initiation step of RNA interference by producing ~22 nt. guide sequences from dsRNAs. Because of its ability to digest dsRNA into uniformly sized, small RNAs, we have named this enzyme Dicer (*Dcr*). *Dicer* mRNA is expressed in embryos, in S2 cells, and in adult flies, consistent with the presence of functional RNAi machinery in all of these contexts (see Supplement 3).

The possibility that Dicer might be the nuclease responsible for the production of guide RNAs from dsRNAs prompted us to raise an antiserum directed against the carboxy-terminus of the Dicer protein (Dicer-1, CG4792). This antiserum could immunoprecipitate a nuclease activity from either Drosophila embryo extracts or from S2 cell lysates that produced ~22 nt. RNAs from dsRNA substrates (Fig. 6C). The putative guide RNAs that are produced by the Dicer-1 enzyme precisely comigrate with 22mers that are produced in extract and with 22mers that are associated with the RISC enzyme (Fig. 6 D,F). It had previously been shown that the enzyme that produced guide RNAs in Drosophila embryo extracts was ATP-dependent (Zamore et al. (2000) Cell 101 25-33). Depletion of this cofactor resulted in an ~6-fold lower rate of dsRNA cleavage and in the production of RNAs with a slightly lower mobility. Of interest was the fact that both Dicer-1 immunoprecipitates and extracts from S2 cells require ATP for the production of ~22mers (Fig. 6D). We do not observe the accumulation of lower mobility products in these cases, although we do routinely observe these in ATP-depleted embryo extracts. The requirement of this nuclease for ATP is a quite unusual property. We hypothesize that this requirement could indicate that the enzyme may act processively on the dsRNA, with the helicase domain harnessing the energy of ATP hydrolysis both for unwinding guide RNAs and for translocation along the substrate.

Efficient induction of RNA interference in *C. elegans* and in *Drosophila* has several requirements. For example, the initiating RNA must be double-stranded, and it must be several hundred nucleotides in length. To determine whether these requirements are dictated by Dicer, we characterized the ability of extracts and of immunoprecipitated enzyme to digest various RNA substrates. Dicer was inactive against single stranded RNAs regardless of length (see Supplement 4). The enyzme could digest both 200 and 500 nucleotide dsRNAs but was significantly less active with shorter substrates (see Supplement 4). Double-stranded RNAs as short as 35 nucleotides could be cut by the

enzyme, albeit very inefficiently (data not shown). In contrast, E. coli RNAse III could digest to completion dsRNAs of 35 or 22 nucleotides (not shown). This suggests that the substrate preferences of the Dicer enzyme may contribute to but not wholly determine the size dependence of RNAi.

To determine whether the Dicer enzyme indeed played a role in RNAi *in vivo*, we sought to deplete Dicer activity from S2 cells and test the effect on dsRNA-induced gene silencing. Transfection of S2 cells with a mixture of dsRNAs homologous to the two Drosophila Dicer genes (CG4792 and CG6493) resulted in an ~6-7 fold reduction of Dicer activity either in whole cell lysates or in Dicer-1 immunoprecipitates (Fig. 7A,B). Transfection with a control dsRNA (murine caspase 9) had no effect. Qualitatively similar results were seen if Dicer was examined by Northern blotting (not shown). Depletion of Dicer in this manner substantially compromised the ability of cells to silence subsequently an exogenous, GFP transgene by RNAi (Fig. 7C). These results indicate that Dicer is involved in RNAi *in vivo*. The lack of complete inhibition of silencing could result from an incomplete suppression of Dicer (which is itself required for RNAi) or could indicate that *in vivo*, guide RNAs can be produced by more than one mechanism (e.g. through the action of RNA-dependent RNA polymerases).

Our results indicate that the process of RNA interference can be divided into at least two distinct steps. According to this model, initiation of PTGS would occur upon processing of a double-stranded RNA by Dicer into ~22 nucleotide guide sequences, although we cannot formally exclude the possibility that another, Dicer-associated nuclease may participate in this process. These guide RNAs would be incorporated into a distinct nuclease complex (RISC) that targets single-stranded mRNAs for degradation. An implication of this model is that guide sequences are themselves derived directly from the dsRNA that triggers the response. In accord with this model, we have demonstrated that ^{32}P-labeled, exogenous dsRNAs that have been introduced into S2 cells by transfection are incorporated into the RISC enzyme as 22 mers (Fig. 7E). However, we cannot exclude the possibility that RNA-dependent RNA polymerases might amplify 22mers once they have been generated or provide an alternative method for producing guide RNAs.

The structure of the Dicer enzyme provokes speculation on the mechanism by which the enzyme might produce discretely sized fragments irrespective of the sequence of the dsRNA (see Supplement 1, Fig. 8a). It has been established that bacterial RNAse III acts on its substrate as a dimer (Nicholson (1999) FEMS Microbiol Rev 23: 371-390; Robertson et al. (1968) J Biol Chem 243: 82-91; Dunn (1976) J Biol Chem 251: 3807-3814). Similarly, a dimer of Dicer enzymes may be required for cleavage of dsRNAs into ~22 nt. pieces. According to one model, the cleavage interval would be determined by the physical arrangement of the two RNAse III domains within Dicer enzyme (Fig. 8a). A

plausible alternative model would dictate that cleavage was directed at a single position by the two RIII domains in a single Dicer protein. The 22 nucleotide interval could be dictated by interaction of neighboring Dicer enzymes or by translocation along the mRNA substrate. The presence of an integral helicase domain suggests that the products of Dicer cleavage might be single-stranded 22 mers that are incorporated into the RISC enzyme as such.

A notable feature of the Dicer family is its evolutionary conservation. Homologs are found in *C. elegans* (K12H4.8), *Arabidopsis* (e.g., CARPEL FACTORY (Jacobson et al. (1999) Development 126: 5231-5243), T25K16.4, AC012328_1), mammals (Helicase-MOI (Matsuda et al. (2000) Biochim Biophys Acta 1490: 163-169) and *S. pombe* (YC9A_SCHPO) (Fig 8b, see Supplements 6,7 for sequence comparisons). In fact, the human Dicer family member is capable of generating ~22 nt. RNAs from dsRNA substrates (Supplement 5) suggesting that these structurally similar proteins may all share similar biochemical functions. It has been demonstrated that exogenous dsRNAs can affect gene function in early mouse embryos (Wianny et al. (2000) Nature Cell Biology 2: 70-75), and our results suggest that this regulation may be accomplished by an evolutionarily conserved RNAi machinery.

In addition to RNAseIII and helicase motifs, searches of the PFAM database indicate that each Dicer family member also contains a ZAP domain (Fig 8c) (Sonnhammer et al. (1997) Proteins 28: 405-420). This sequence was defined based solely upon its conservation in the Zwille/ARGONAUTE/Piwi family that has been implicated in RNAi by mutations *in C. elegans* (Rde-1) and *Neurospora* (Qde-2) (Tabara et al. (1999) Cell 99: 123-132; Catalanotto et al (2000) Nature 404: 245). Although the function of this domain is unknown, it is intriguing that this region of homology is restricted to two gene families that participate in dsRNA-dependent silencing. Both the ARGONAUTE and Dicer families have also been implicated in common biological processes, namely the determination of stem-cell fates. A hypomorphic allele of *carpel factory*, a member of the Dicer family in *Arabidopsis*, is characterized by increased proliferation in floral meristems (Jacobsen et al. (1999) Development 126 5231-5243). This phenotype and a number of other characteristic features are also shared by *Arabidopsis ARGONAUTE* (*ago1-1*) mutants (Bohmert et al. (1998) EMBO J 17: 170-180; C. Kidner and R. Martiennsen, pers. comm.). These genetic analyses begin to provide evidence that RNAi may be more than a defensive response to unusual RNAs but may also play important roles in the regulation of endogenous genes.

With the identification of Dicer as a catalyst of the initiation step of RNAi, we have begun to unravel the biochemical basis of this unusual mechanism of gene regulation. It will be of critical importance to determine whether the conserved family

members from other organisms, particularly mammals, also play a role in dsRNA-mediated gene regulation.

Methods

Plasmid constructs. A full-length cDNA encoding Drosha was obtained by PCR from an EST sequenced by the Berkeley Drosophila genome project. The *Homeless* clone was a gift from Gillespie and Berg (Univ. Washington). The T7 epitope-tag was added to the amino terminus of each by PCR, and the tagged cDNAs were cloned into pRIP, a retroviral vector designed specifically for expression in insect cells (E. Bernstein, unpublished). In this vector, expression is driven by the *Orgyia pseudotsugata* IE2 promoter (Invitrogen). Since no cDNA was available for CG4792/Dicer, a genomic clone was amplified from a bacmid (BACR23F10; obtained from the BACPAC Resource Center in the Dept. of Human Genetics at the Roswell Park Cancer Institute). Again, during amplification, a T7 epitope tag was added at the amino terminus of the coding sequence. The human Dicer gene was isolated from a cDNA library prepared from HaCaT cells (GJH, unpublished). A T7-tagged version of the complete coding sequence was cloned into pCDNA3 (Invitrogen) for expression in human cells (LinX-A).

Cell culture and extract preparation. *S2 and embryo culture.* S2 cells were cultured at 27°C in 5% CO_2 in Schneider's insect media supplemented with 10% heat inactivated fetal bovine serum (Gemini) and 1% antibiotic-antimycotic solution (Gibco BRL). Cells were harvested for extract preparation at $10x10^6$ cells/ml. The cells were washed 1X in PBS and were resuspended in a hypotonic buffer (10 mM Hepes pH 7.0, 2 mM $MgCl_2$, 6 mM βME) and dounced. Cell lysates were spun 20,000xg for 20 minutes. Extracts were stored at –80°C. *Drosophila* embryos were reared in fly cages by standard methodologies and were collected every 12 hours. The embryos were dechorionated in 50% chlorox bleach and washed thoroughly with distilled water. Lysis buffer (10 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM DTT, 0.2 mM PMSF) was added to the embryos, and extracts were prepared by homogenization in a tissue grinder. Lysates were spun for two hours at 200,000xg and were frozen at –80°C. LinX-A cells, a highly-transfectable derivative of human 293 cells, (Lin Xie and GJH, unpublished) were maintained in DMEM/10% FCS.

Transfections and immunoprecipitations. S2 cells were transfected using a calcium phosphate procedure essentially as previously described (Hammond et al. (2000) Nature 404: 293-296). Transfection rates were ~90% as monitored in controls using an *in situ* β-galactosidase assay. LinX-A cells were also transfected by calcium phosphate co-precipitation. For immunoprecipitations, cells (~ $5x10^6$ per IP) were transfected with various clones and lysed three days later in IP buffer (125 mM KOAc, 1 mM MgOAc, 1

mM $CaCl_2$, 5 mM EGTA, 20 mM Hepes pH 7.0, 1 mM DTT, 1% NP-40 plus Complete protease inhibitors (Roche)). Lysates were spun for 10 minutes at 14,000xg and supernatants were added to T7 antibody-agarose beads (Novagen). Antibody binding proceeded for 4 hours at 4°C. Beads were centrifuged and washed in lysis buffer three times, and once in reaction buffer. The Dicer antiserum was raised in rabbits using a KLH-conjugated peptide corresponding to the C-terminal 8 amino acids of Drosophila Dicer-1 (CG4792).

Cleavage reactions. *RNA preparation.* Templates to be transcribed into dsRNA were generated by PCR with forward and reverse primers, each containing a T7 promoter sequence. RNAs were produced using Riboprobe (Promega) kits and were uniformly labeling during the transcription reaction with ^{32}P-UTP. Single-stranded RNAs were purified from 1% agarose gels. *dsRNA cleavage.* Five microliters of embryo or S2 extracts were incubated for one hour at 30°C with dsRNA in a reaction containing 20 mM Hepes pH 7.0, 2 mM MgOAc, 2 mM DTT, 1 mM ATP and 5% Superasin (Ambion). Immunoprecipitates were treated similarly except that a minimal volume of reaction buffer (including ATP and Superasin) and dsRNA were added to beads that had been washed in reaction buffer (see above). For ATP depletion, *Drosophila* embryo extracts were incubated for 20 minutes at 30°C with 2mM glucose and 0.375 U of hexokinase (Roche) prior to the addition of dsRNA.

Northern and Western analysis. Total RNA was prepared from *Drosophila* embryos (0-12 hour), from adult flies, and from S2 cells using Trizol (Lifetech). Messenger RNA was isolated by affinity selection using magnetic oligo-dT beads (Dynal). RNAs were electrophoresed on denaturing formaldehyde/agarose gels, blotted and probed with randomly primed DNAs corresponding to Dicer. For Western analysis, T7-tagged proteins were immunoprecipitated from whole cell lysates in IP buffer using anti-T7-antibody-agarose conjugates. Proteins were released from the beads by boiling in Laemmli buffer and were separated by electrophoresis on 8% SDS PAGE. Following transfer to nitrocellulose, proteins were visualized using an HRP-conjugated anti-T7 antibody (Novagen) and chemiluminescent detection (Supersignal, Pierce).

RNAi of Dicer. Drosophila S2 cells were transfected either with a dsRNA corresponding to mouse caspase 9 or with a mixture of two dsRNAs corresponding to Drosophila Dicer-1 and Dicer-2 (CG4792 and CG6493). Two days after the initial transfection, cells were again transfected with a mixture containing a GFP expression plasmid and either luciferase dsRNA or GFP dsRNA as previously described (Hammond et al. (2000) Nature 404: 293-296). Cells were assayed for Dicer activity or fluorescence three days after the second transfection. Quantification of fluorescent cells was done on a

Coulter EPICS cell sorter after fixation. Control transfections indicated that Dicer activity was not affected by the introduction of caspase 9 dsRNA.

Example 3: A Simplified Method for the Creation of Hairpin Constructs for RNA Interference.

In numerous model organisms, double stranded RNAs have been shown to cause effective and specific suppression of gene function (Bosher and Labouesse (2000) Nature Cell Biology 2: E31-E36). This response, termed RNA interference or post-transcriptional gene silencing, has evolved into a highly effective reverse genetic tool in *C. elegans*, *Drosophila*, plants and numerous other systems. In these cases, double-stranded RNAs can be introduced by injection, transfection or feeding; however, in all cases, the response is both transient and systemic. Recently, stable interference with gene expression has been achieved by expression of RNAs that form snap-back or hairpin structures (Fortier and Belote (2000) Genesis 26: 240-244; Kennerdell and Carthew (2000) Nature Biotechnology 18: 896-898; Lam and Thummel (2000) Current Biology 10: 957-963; Shi et al. (2000) RNA 6: 1069-1076; Smith et al. (2000) Nature 407: 319-320; Tavernarakis et al. (2000) Nature Genetics 24: 180-183). This has the potential not only to allow stable silencing of gene expression but also inducible silencing as has been observed in trypanosomes and adult *Drosophila* (Fortier and Belote (2000) Genesis 26: 240-244; Lam and Thummel (2000) Current Biology 10: 957-963; Shi et al. (2000) RNA 6: 1069-1076). The utility of this approach is somewhat hampered by the difficulties that arise in the construction of bacterial plasmids containing the long inverted repeats that are necessary to provoke silencing. In a recent report, it was stated that more than 1,000 putative clones were screened to identify the desired construct (Tavernarakis et al. (2000) Nature Genetics 24: 180-183).

The presence of hairpin structures often induces plasmid rearrangement, in part due to the E. coli sbc proteins that recognize and cleave cruciform DNA structures (Connelly et al. (1996) Genes Cell 1: 285-291). We have developed a method for the construction of hairpins that does not require cloning of inverted repeats, per se. Instead, the fragment of the gene that is to be silenced is cloned as a direct repeat, and the inversion is accomplished by treatment with a site-specific recombinase, either *in vitro* (or potentially *in vivo)* (see Fig 27). Following recombination, the inverted repeat structure is stable in a bacterial strain that lacks an intact SBC system (DL759). We have successfully used this strategy to construct numerous hairpin expression constructs that have been successfully used to provoke gene silencing in Drosophila cells.

In the following examples, we use this method to express long dsRNAs in a variety of mammalian cell types. We show that such long dsRNAs mediate RNAi in a variety of cell types. Additionally, since the vector described in Figure 27 contains a selectable marker, dsRNAs produced in this manner can be stably expressed in cells. Accordingly, this method allows not only the examination of transient effects of RNA suppression in a cell, but also the effects of stable and prolonged RNA suppression.

Methods:

Plasmids expressing hairpin RNAs were constructed by cloning the first 500 basepairs of the GFP coding region into the FLIP cassette of pRIP-FLIP as a direct repeat. The FLIP cassette contains two directional cloning sites, the second of which is flanked by LoxP sites. The Zeocin gene, present between the cloning sites, maintains selection and stability. To create an inverted repeat for hairpin production, the direct repeat clones were exposed to Cre recombinase (Stratagene) in vitro and, afterwards, transformed into DL759 E.Coli. These bacteria permit the replication of DNA containing cruciform structures, which tend to form inverted repeats.

Example 4: Long dsRNAs Suppress Gene Expression in Mammalian Cells

Previous experiments have demonstrated that dsRNA, produced using a variety of methods including via the construction of hairpins, can suppress gene expression in Drosophila cells. We now demonstrate that dsRNA can also suppress gene expression in mammalian cells in culture. Additionally, we demonstrate that RNA suppression can be mediated by stably expressing a long hairpin in a mammalian cell line. The ability to engineer stable silencing of gene expression in cultured mammalian cells, in addition to the ability to transiently silence gene expression, has many important applications.

A. Figure 28 shows wildtype P19 cells which have been co-transfected with either RFP or GFP (Figure 28, right panel). Note the robust expression of RFP or GFR respectively approximately 42 hours post-transfection. We isolated P19 clones which stably express a 500 nt. GFP hairpin. Such clones were then transfected with either RFP or GFP, and expression of RFP or GFP was assessed by visual inspection of the cells. The left panel of Figure 28 demonstrates that a 500 nt GFP hairpin specifically suppresses expression of GFP in P19 cells.

B. Similar experiments were performed using several cell lines in order to demonstrate that dsRNA can suppress gene expression generally in mammalian cells. Figure 29 shows the results of a transient co-transfection assay performed in Hela cells, CHO cells and P19 cells. The cell lines were each transfected with plasmids expressing *Photinus pyralis* (firefly) and *Renila reniformis* (sea pansy) luciferases. The cells lines were additionally transfected with 400 ng of 500nt dsRNAs corresponding to either firefly luciferase (dsLUC) or dsGFP. Dual luciferase assays were carried out using an Analytical Scientific Instruments model 3010 Luminometer. Renilla luciferase serves as an internal control for dsRNA specific suppression of firefly luciferase activity. All values are normalized to dsGFP control.

The results summarized in Figure 29 demonstrate that dsRNA can specifically mediate suppression in a multiple mammalian cells types in culture. Additionally, such experiments were successfully carried out in mouse ES cells (Figure 30). Our ability to successfully manipulate ES cell via RNAi allows the use of RNAi in the generation of transgenic and knock-out mice.

C. Figure 31 demonstrates that dsRNA can mediate suppression of gene expression in mammalian cells, and that this suppression is stable over time. Experiments were carried out largely as described in part B. Briefly, P19 cells were transfected with plasmids expressing *Photinus pyralis* (firefly) and *Renilla reniformis* (sea pansy) luciferases, and 500 nt dsRNA corresponding to either firefly luciferase or to GFP. Dual luciferase assays were carried out using an Analytical Scientific Instruments model 3010 Luminometer.

The results summarized in Figure 31 demonstrate that dsRNA can mediate suppression in mammalian cells in culture, and that this suppression is stable over time. A comparable level of suppression of firefly gene expression was observed at 12 hours, 24 hours, and 50 hours post-transfection.

D. Although the above experiments demonstrate the ability to suppress gene expression in mammalian cells using dsRNA, such experiments do not address the mechanisms by which such suppression occurs. To begin to address whether dsRNA mediated suppression of gene expression in mammalian cells is mechanistically similar to dsRNA suppression in invertebrates, we examined the ability of the 500nt dsRNA constructs described above to suppress gene expression in vitro in extracts from P19 cells.

S10 fractions from P19 cell lysates were used for in vitro translation of mRNA encoding *Photinus pyralis* (firefly) and *Renilla reniformis* (sea pansy) luciferases. dsRNA

corresponding to firefly luciferase or to GFP was added to the reactions. Following reactions performed at 30 °C for 1 hour, dual luciferase assays were performed using an Analytical Scientific Instruments model 3010 Luminometer.

Figure 32 summarizes the results of these experiments which demonstrate that dsRNA can specifically suppress gene expression in an in vitro mammalian cell system in a manner which is consistent with post-transcriptional gene silencing.

E. To further confirm that the dsRNA mediated suppression observed was consistent with post-transcriptional gene silencing, we examined RNA suppression in the absence of Dicer expression. As detailed herein, Dicer has been identified as an important factor in post-transcriptional gene silencing. Accordingly, if the effects described here are consistent with our understanding of post-transcriptional gene silencing, then you would not expect robust and specific suppression to occur in the absence of Dicer expression.

Figure 33 summarizes these results. Briefly, P19 cells stably expressing the long dsRNA for GFP were transfected with either GFP or with GFP plus dsDicer RNA. The top panels demonstrate that stably expressed long dsRNA to GFP specifically suppresses GFP expression in P19 cells (as detailed in previous examples). However, in the presence of dsDicer RNA, GFP expression is observed in these cells.

This experiment provides further evidence indicating that the RNA mediated suppression observed upon stable expression of long dsRNAs functions by a mechanism consistent with post-transcriptional gene silencing.

F. Although the results summarized in Figures 32-33 appear to demonstrate that dsRNA can specifically suppress gene expression in a manner consistent with post-transcriptional silencing, we wanted to verify that the suppressive effects observed in the in vitro system were specific to double stranded RNA.

Briefly, experiments were performed in accordance with the methods outlined above. Either dsRNA (ds), single-stranded RNA (ss), or antisense-RNA (as) corresponding to firefly (FF) or *Renilla* (Ren) luciferase was added to the translation reaction. Following reactions performed at 30 °C for 1 hour, dual luciferase assays were performed using an Analytical Scientific Instruments model 3010 Luminometer.

Figure 34 summarizes the results of these experiments which demonstrate that the suppression of gene expression observed in this in vitro assay is specific for dsRNA. These results further support the conclusion that dsRNA suppresses gene expression in this mammalian in vitro system in a manner consistent with post-transcriptional silencing.

G. Studies of post-transcriptional silencing in invertebrates have demonstrated that transfection or injection of the dsRNA is not necessary to achieve the suppressive affects. For example, dsRNA suppression in C. elegans can be observed by either soaking the worms in dsRNA, or by feeding the worms bacteria expressing the dsRNA of interest. We addressed whether dsRNA suppression in mammalian cells could be observed without transfection of the dsRNA. Such a result would present additional potential for easily using dsRNA suppression in mammalian cells, and would also allow the use of dsRNA to suppress gene expression in cell types which have been difficult to transfect (i.e., cell types with a low transfection efficiency, or cell types which have proven difficult to transfect at all).

P19 cells were grown in 6-well tissue culture plates to approximately 60% confluency in growth media (αMEM/10% FBS). Varying concentrations of firefly dsRNA were added to the cultures, and cells were cultured for 12 hours in growth media + dsRNA. Cells were then transfected with plasmids expressing firefly or sea pansy luciferase, as described in detail above. Dual luciferase assays were carried out 12 hours post-transfection using an Analytical Scientific Instruments model 3010 Luminometer.

Figure 35 summarizes these results which demonstrate that dsRNA can suppress gene expression in mammalian cells without transfection. Culturing cells in the presence of dsRNA resulted in a dose dependent suppression of firefly luciferase gene expression.

Example 5: Compositions and Methods for Synthesizing siRNAs

Previous results have indicated that short synthetic RNAs (siRNAs) can efficiently induce RNA suppression. Since short RNAs do not activate the non-specific PKR response, they offer a means for efficiently silencing gene expression in a range of cell types. However, the current state of the art with respect to siRNAs has several limitations. Firstly, siRNAs are currently chemically synthesized at great cost (approx. $400/siRNA). Such high costs make siRNAs impractical for either small laboratories or for use in large scale screening efforts. Accordingly, there is a need in the art for methods for generating siRNAs at reduced cost.

We provide compositions and methods for synthesizing siRNAs by T7 polymerase. This approach allows for the efficient sythesis of siRNAs at a cost consistent with standard RNA transcription reactions (approx. $16/siRNA). This greatly reduced cost makes the use of siRNA a reasonable approach for small laboratories, and also will facilitate their use in large-scale screening projects.

Figure 36 shows the method for producing siRNAs using T7 polymerase. Briefly, T7 polymerase is used to transcribe both a sense and antisense transcript. The transcripts are then annealed to provide an siRNA. One of skill in the art will recognize that any one of the available RNA polymerases can be readily substituted for T7 to practice the invention (i.e., T3, Sp6, etc.).

This approach is amenable to the generation of a single siRNA species, as well as to the generation of a library of siRNAs. Such a library of siRNAs can be used in any number of high-throughput screens including cell based phenotypic screens and gene array based screens.

Example 6: Generation of Short Hairpin dsRNA and Suppression of Gene Expression Using Such Short Hairpins

We have generated several types of short dsRNAs corresponding to the coding region of firefly or Renilla luciferase (as outlined in detail above for long dsRNAs). Accordingly, the specificity of short dsRNAs in suppressing gene expression can be evaluated in much the same way the specificity of long dsRNAs was evaluated. Figure 37 details the structure of three types of short dsRNAs tested for their efficacy in specifically suppressing gene expression in cell culture. The three basic types of short RNAs are siRNAs, let-7 like hairpin RNAs, and simple hairpins.

A. The ability of short dsRNAs to specifically suppress gene expression was analyzed in Drosophila S2 cells. Figure 38 summarizes experiments which demonstrate that short hairpins corresponding to firefly luciferase specifically suppress firefly luciferase gene expression. All three types of short dsRNAs (siRNA, let-7 like hairpins, and simple hairpins) dramatically and specifically suppress gene expression in comparison to Renilla luciferase control RNAs. Note that the siRNA and the simple hairpin appear to suppress gene expression a little more effectively than the let-7 like hairpin.

B. Figure 39 summarizes experiments which demonstrate that short dsRNAs corresponding to firefly luciferase specifically suppress gene expression in human 293T cells. All three types of short dsRNAs (siRNA, let-7 like hairpins, and simple hairpind) dramatically and specifically suppress gene expression in comparison to Renilla luciferase control RNAs. Note however, consistant with the results observed in Drosophila S2 cells, the siRNA and the simple hairpin appear to suppress gene expression a more effectively than the let-7 like hairpin.

C. Figure 39 demonstrates that several types of short dsRNAs can specifically suppress gene expression in mammalian cells. We wanted to confirm that short dsRNAs can suppress gene expression in other mammalian cells. Additionally, we wanted to demonstrate that unlike long dsRNAs, short dsRNAs do not provoke a non-specific PKR or PKR-like response. Figure 40 summarizes experiments performed in HeLa cells which demonstrate that short dsRNAs specifically suppress gene expression in HeLa cells. The specific suppression observed in HeLa cells in the presence of short dsRNAs is contrary to the non-specific effects observed when HeLa cells were treated with long dsRNAs, and demonstrate that short dsRNAs do not provoke a non-specific PKR or PKR-like response.

D. In an attempt to further understand the mechanisms by which short hairpins suppress gene expression, we examined the effects of transfecting cells with a mixture of two different short hairpins corresponding to firefly luciferase. Figure 41 summarizes the results of experiments which suggest that there is no synergistic affects on suppression of firefly luciferase gene expression obtained when cells are exposed to a mixture of such short hairpins.

Example 7: Encoded Short Hairpins Function In Vivo

An object of the present invention is to improve methods for generating siRNAs and short hairpins for use in specifically suppressing gene expression. Example 6 demonstrates that siRNAs and short hairpins are highly effective in specifically suppressing gene expression. Accordingly, it would be advantageous to combine the efficient suppression of gene expression attainable using short hairpins and siRNAs with a method to encode such RNA on a plasmid and express it either transiently or stably.

Figure 42 demonstrates that short hairpins encoded on a plasmid are effective in suppressing gene expression. DNA oligonucleotides encoding 29 nucleotide hairpins corresponding to firefly luciferase were inserted into a vector containing the U6 promoter. Three independent constructs were examined for their ability to specifically suppress firefly luciferase gene expression in 293T cells. siOligo1-2, siOligo1-6, and siOligo1-19 (construct in the correct orientation) each suppressed gene expression as effectively as siRNA. In contrast, siOligo1-10 (construct in the incorrect orientation) did not suppress gene expression. Additionally, an independent construct targeted to a different portion of the firefly luciferase gene did not effectively suppress gene expression in either orientation (siOligo2-23, siOligo2-36).

The results summarized in Figure 42 demonstrate that transient expression of siRNAs and short hairpins encoded on a plasmid can efficiently suppress gene expression. One of skill can choose from amongst a range of vectors to either transiently or stably express an siRNA or short hairpin. Non-limiting examples of vectors and strategies to stably express short dsRNAs are presented in Figures 43-45.

Example 8: dsRNA Suppression in the Absence of a PKR Response

One potential impediment to the use of RNAi to suppress gene expression in some cell types, is the non-specific PKR response that can be triggered by long dsRNAs. Numerous mammalian viruses have evolvd the ability to block PKR inorder to aid in the infection of potential host cells. For example, adenoviruses express RNAs which mimic dsRNA but do not activate the PKR response. Vaccinia virus uses two strategies to evade PKR: the expression of E3L which binds and masks dsRNA; the expression of K3L to mimic the natural PKR substrate eIF2α.

Our understanding of the mechanisms by which viruses avoid the PKR response allows us to design approaches to circumvent the PKR response in cell types in which in might be advantageous to suppression gene expression with long dsRNAs. Possible approaches include treating cells with an agent that inhibits protein kinase RNA-activated (PKR) apoptosis, such as by treatment with agents which inhibit expression of PKR, cause its destruction, and/or inhibit the kinase activity of PKR. Accordingly, RNAi suppression of gene expression in such cell types could involve first inhibiting the PKR response, and then delivering a dsRNA identical or similar to a target gene.

A. In a murine myoblast cell line, C2C12, we noted that the cells responded to long dsRNAs with a mixture of specific and non-specific (presumably PKR) responses. In order to attenuate the non-specific PKR response while maintaining the robust and specific suppression due to the long dsRNA, C2C12 cells were transfected with a vector that directs K3L expression. This additional step successfully attenuated the PKR response, however expression of K3L protein had no effect on the magnitude of specific inhibition.

B. However, since the efficacy of such a two step approach had not been previously demonstrated, it was formerly possible that dsRNA suppression would not be possible in cells with a PKR response. Figure 46 summarizes results which demonstrate that such a two step approach is possible, and that robust and specific dsRNA mediated suppression is possible in cells which had formerly possessed a robust PKR response.

Briefly, dual luciferase assay were carried out as described in detail above. The experiments were carried out using PKR -/- MEFs harvested from E13.5 PKR-/- mosue embryos. MEFs typically have a robust PKR response, and thus treatment with long dsRNAs typically results in non-specific suppression of gene expression and apoptosis. However, in PKR -/- cells examined 12, 42, and 82 hours after transfection, expression of ds*Renilla* luciferase RNA specifically suppresses expression *Renilla reniformis* (sea pansy) luciferase. This suppression is stable over time.

These results demonstrate that the non-specific PKR response can be blocked without affecting specific suppression of gene expression mediated by dsRNA. This allows the use of long dsRNAs to suppress gene expression in a diverse range of cell types, including those that would be previously intractable due to the confounding influences of the non-specific PKR response to long dsRNA.

Example 9: Suppression of Gene Expression using dsRNA which Corresponds to Non-Coding Sequence

Current models for the mechanisms which drive RNAi have suggested that the dsRNA construct must contain coding sequence corresponding to the gene of interest. Although evidence has demonstrated that such coding sequence need not be a perfect match to the endogenous coding sequence (i.e., it may be similar), it has been widely held that the dsRNA construct must correspond to coding sequence. We present evidence that contradicts the teachings of the prior art, and demonstrate that dsRNA corresponding to non-coding regions of a gene can suppress gene function in vivo. These results are significant not only because they demonstrate that dsRNA identical or similar to non-coding sequences (i.e., promoter sequences, enhancer sequences, or intronic sequences) can mediate suppression, but also because we demonstrate the in vivo suppression of gene expression using dsRNA technology in a mouse model.

We generated doubled stranded RNA corresponding to four segments of the mouse tyrosinase gene promoter. Three of these segments correspond to the proximal promoter and one corresponds to an enhancer (Fig. 47). The tyrosinase gene encodes the rate limiting enzyme involved in the melanin biosynthetic pathway (Bilodeau et al. (2001) Pigment Cell Research 14: 328-336). Accordingly, suppression of the tyrosinase gene is expected to inhibit pigmentation.

Double stranded RNA corresponding to each of the above promoter segments was injected into the pronuclei of fertilized eggs. Pups were born after 19 days. In total

42/136 (31%) of the embryos were carried to term. This number is within the expected range for transgenesis (30-40%). Two pups out of 42 (5%) appear totally unpigmented at birth, consistent with suppression of tyrosinase function.

Methods:

dsRNA from non-coding promoter region of tyrosinase gene. Four segments of the mouse tyrosinase gene promoter were amplified by PCR using primers which incorporated T7 RNA polymerase promoters into the PCR products (**shown in bold** – Fig. 47). Sequences of the mouse tyrosinase gene 5' flanking regions were obtained from GenBank (accession number D00439 and X51743). The sequence of the tyrosinase enhancer, located approximately 12 kb upstream of the transcriptional start site, was also obtained from GenBank (accession number X76647).

The sequences of the primers used were as follows: note the sequence of the T7 RNA polymerase promoter is shown in bold

Tyrosinase enhancer (~12 kb upstream)

5' **TAATACGACTCACTATAGGG**CAAGGTCATAGTTCCTGCCAGCTG 3'

5' **TAATACGACTCACTATAGGG**CAGATATTTTCTTACCACCCACCC 3'

-1404 to –1007

5' **TAATACGACTCACTATAGGG**TTAAGTTTAACAGGAGAAGCTGGA 3'

5' **TAATACGACTCACTATAGGG**AAATCATTGCTTTCCTGATAATGC 3'

-1003 to –506

5' **TAATACGACTCACTATAGGG**TAGATTTCCGCAGCCCCAGTGTTC 3'

5' **TAATACGACTCACTATAGGGG**TTGCCTCTCATTTTTCCTTGATT 3'

-505 to –85

5' **TAATACGACTCACTATAGGG**TATTTTAGACTGATTACTTTTATAA 3'

5' **TAATACGACTCACTATAGGG**TCACATGTTTTGGCTAAGACCTAT 3'

PCR products were gel purified from 1% TAE agarose gels using QiaExII Gel Extraction Kit (Qiagen). Double stranded RNA was produced from these templates using T7-Megashortscript Kit (Ambion). Enzymes and unincorporated nucleotides were removed using Qiaquick MinElute PCR Purification Kit. RNA was phenol/chloroform extracted twice, and ethanol precipitated. Pellets were resuspended in injection buffer ((10 mM Tris (pH 7.5), 0.15 nM EDTA (pH 8.0)) at a concentration of 20 ng/ul and run on a 1% TAE agarose gel to confirm integrity.

Generation of mice: An equal mixture of double stranded RNA from each of the above primer sets was injected into the pronuclei of fertilized eggs from C57BL6J mice. A total of 136 injections was performed, and 34 embryos were implanted into each of 4 pseudopregnant CD-1 females. Pups were born after 19 days. In total, 42/136 (31%) of the embryos were carried to term. 2/42 pups (5%) appear totally unpigmented at birth.

It is not clear whether the RNAi mediated by dsRNA identical or similar to non-coding sequence works via the same mechanism as PTGS observed in the presence of dsRNA identical or similar to coding sequence. However, whether these results ultimately reveal similar or differing mechanisms does not diminish the tremendous utility of the compositions and methods of the present invention to suppress expression of one or more genes in vitro or in vivo.

The present invention demonstrates that dsRNA ranging in length from 20-500 nt can readily suppress expression of target genes both in vitro and in vivo. Furthermore, the present invention demonstrates that the dsRNAs can be generated using a variety of methods including the formation of hairpins, and that these dsRNAs can be expressed either stably or transiently. Finally, the present invention demonstrates that dsRNA identical or similar to non-coding sequences can suppress target gene expression.

V. Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

All of the above-cited references and publications are hereby incorporated by reference.